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THE ROLE OF A PANEL OF THREE EPIGENETIC BIOMARKERS IN THE DETECTION AND PROGNOSIS OF UPPER URINARY TRACT UROTHELIAL CARCINOMA

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“Even if I knew that tomorrow the world would go to pieces,
I would still plant my apple tree”.

Martin Luther

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SUMMARY

BACKGROUND: Upper tract urothelial carcinoma (UTUC) accounts for 5-10% of all urothelial tumors. It is mostly diagnosed at advanced stages, entailing a worse prognosis, owing to the lack of early and specific symptoms as well as of effective diagnostic tools.

AIMS: We previously identified a panel of epigenetic biomarkers (*GDF15*, *TMEFF2* and *VIM* promoter methylation) that accurately identifies bladder cancer in urine. Herein, the main aims of this Thesis were to assess the performance of the same panel for UTUC detection and prognosis, in tissue and urine, and to investigate its relevance in the urothelial carcinogenesis processes.

MATERIAL AND METHODS: Methylation levels of reference and target genes were determined using real-time quantitative MSP in bisulfite-modified DNA of 57 UTUC tissues, 36 normal upper tract urothelium (NUTUs), 22 urines from UTUC suspects and 20 urines from controls.
ROC-curve analysis was performed to determine the performance of the biomarker panel and survival analyses were conducted to evaluate their prognostic value.
Quantitative gene expression analysis, western blot and ChIP were performed to evaluate VIM deregulation in transitional cell lines exposed to DAC and TSA.

RESULTS: Methylation levels of *GDF15*, *TMEFF2* and *VIM* were significantly higher in UTUC compared to NUTUs ($P = 0.022$; $P < 0.001$; $P < 0.001$, respectively). The panel accurately identified UTUC with 100% and 91% sensitivity, corresponding to an area under the curve of 1.000 and 0.923 in tissue and urines, respectively, with 100% specificity. Low *VIM* promoter methylation levels independently predicted poor disease-specific survival.
VIM transacript and protein levels increased after exposure to DAC, and for the same conditions, it was observed an increase of deposition of histone activation marks, with concomitant decrease of a repressive mark.

CONCLUSIONS: *GDF15, TMEFF2* and *VIM* promoter methylation allows for accurate identification of UTUC, in tissue and urine, and *VIM* methylation provide relevant prognostic information, especially in high-stage disease. Because UTUC are

difficult to detect clinically and imagiologically at an early stage, this gene panel might provide a useful tool for early detection of UTUC in urine samples, and improve the clinical management of UTUC patients. Moreover, we confirmed that *VIM* is epigenetically regulated in transitional carcinoma cell lines, namely through the interaction of promoter DNA methylation and histone post-translational modifications.

RESUMO

INTRODUÇÃO: 5 a 10% de todas as neoplasias uroteliais são devidas ao carcinoma urotelial do trato urinário superior (UTUC). Este ultimo é sobretudo diagnosticado em estádios avançados de doença, tendo assim um pior prognóstico, muito devido ao facto de os principais sintomas serem praticamente nulos em estádios iniciais da doença, e também pela falta de testes de diagnóstico realmente eficazes.

OBJECTIVOS: Recentemente, o nosso grupo de investigação identificou um painel de biomarcadores epigenéticos (a metilação dos promotores de *GDF15, TMEFF2* e *VIM*) que detecta carcinoma urotelial da bexiga, tanto em amostras de tecido como em amostras de urina. Assim, os principais objectivos desta Tese consistem em avaliar, em tecidos e urinas, a utilidade deste mesmo painel na detecção e prognóstico de UTUC, e investigar a sua relevância nos processos carcinogénese urotelial.

MATERIAL E MÉTODOS: Os níveis de metilação dos genes alvo e de referência foram determinados através de PCR quantitativo de metilação em tempo real utilizando 57 amostras de DNA de tecidos provenientes de UTUC, 36 amostras de urotélio normal do trato urinário superior (NUTU), 22 urinas provenientes de doentes suspeitos de UTUC, e 20 urinas de controlos.
Foi realizada uma análise das curvas ROC para determinar a performance do painel de biomarcadores, bem como análises de sobrevivência para avaliar o seu respectivo valor prognóstico.
Adicionalmente, foram também realizadas análises quantitativas de expressão génica, western blot e ChIP para avaliar a desregulação da VIM em linhas celulares de carcinoma transicional expostas aos agentes DAC e TSA.

RESULTADOS: Os níveis de metilação do *GDF15*, *TMEFF2* e *VIM* estavam significativamente aumentados nos UTUC quando comparados com os NUTU ($P = 0.022$; $P < 0.001$; $P < 0.001$, respectivamente). O mesmo painel conseguiu identificar UTUC em tecidos e urinas com uma sensibilidade de 100% e 91%, respectivamente, correspondente a uma área sob a curva de 1.000 e 0.923, e com uma especificidade de 100%. Os baixos níveis de metilação do promotor do

VIM foram igualmente identificados como marcadores independentes de mau prognóstico.

Os níveis de transcrito e proteína da VIM aumentaram significativamente após a exposição à DAC, e para as mesmas condições, foi observado um aumento da deposição de marcas ativadoras das histonas, com concomitante decréscimo de uma marca repressora.

CONCLUSÕES: A metilação dos promotores de *GDF15, TMEFF2* e *VIM* permite a identificação de UTUC, tanto em tecidos como em urinas, fornecendo informação relevante sobre o prognóstico, principalmente para os estádios mais avançados. Devido às dificuldades na detecção clinica e imagiológica em estádios iniciais da doença, este painel de genes poderá constituir um importante método auxiliar de diagnóstico precoce de UTUC em amostras de urina, com um previsível impacto na decisão clínica.

Finalmente, foi possível confirmar que o promotor do gene *VIM* é regulado epigeneticamente, havendo uma interação entre a metilação do DNA e as modificações pós-transducionais das histonas.

LIST OF ABBREVIATIONS

A	Adenine
ACTB	Beta Actin
AUC	Area Under the Curve
BlCa	Bladder Cancer
C	Cytosine
CpG	Cytosine-phosphate-Guanine
DAC	5'aza-2'-deoxycytidine
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
FAM	6-carboxy-fluorescein
FBS	Fetal Bovine Serum
G	Guanine
GDF15	Growth differentiation factor 15
GUSB	Beta Glucuronidase
H2AZ	Histone 2AZ
H3	Histone 3
H4	Histone 4
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDM	Histone Demethylase
HMT	Histone Methyltransferase
HPRT	Hypoxanthine phosphoribosiltransferase
LOI	Loss of Imprinting
m^5C	5- methylcytosine
MBD	3'-minor groove binder
MSP	Methylation Specific PCR
NPV	Negative Predictive Value
P-S	Penicillin- Streptomycin
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PPV	Positive Predictive Value
qMSP	Quantitative Methylation Specific PCR

RNA	Ribonucleic Acid
ROC	Receiver Operator Characteristics
SAM	S-Adenosyl-Methionine
T	Thymine
TAMRA	6-carboxy-tetramethyl-rhodamine
TMEFF2	Transmembrane Protein with EGF-like and two follistatin-like domains 2
TSA	Trichostatin A
TSG	Tumor Suppressor Gene
U	Uracil
UC	Urothelial Cancer
UTUC	Upper Tract Urothelial Carcinoma
VIM	Vimentin
WHO	World Health Organization

INTRODUCTION

1. Epigenetics

In 1941, C. H. Waddington coined the term *Epigenetics* referring to the study of the "causal mechanisms by which the genes of the genotype bring about phenotypic effects [1], years before DNA was identified as the molecule of inheritance by Watson and Crick. That definition suffered an evolution since then, and nowadays suits better in the field of developmental biology. If we search for the etymological meaning of the word, we found that the Greek prefix *epi-* in *epigenetics* implies features that are "on top of" or "in addition to" genetics; thus *epigenetic* traits exist on top of / in addition to the traditional molecular basis for inheritance. The actual definition of Epigenetics is now less focused on genotype, and more related to mechanisms that controls gene expression in a potentially heritable way, with no change in the primary DNA sequence [2, 3].

1.1. Epigenetic Mechanisms

Epigenetic changes are preserved when cells divide. Most epigenetic changes only occur within the course of one individual organism's lifetime, but, if gene deactivation occurs in a sperm or egg cell that results in fertilization, then some epigenetic changes can be transferred to the next generation [4].

As previously mentioned, epigenetic changes are capable of modifying the expression of certain genes, but not the DNA sequence itself; in addition, the chromatin proteins associated with DNA may be activated or silenced. Due to these phenomena, a multi-cellular organism can be comprised of several differentiated cells owing the same genetic sequence, but expressing only the genes that are necessary for their own activity. The concept of *Epigenome* fits in these findings: having a group of molecular mechanisms that act together towards the regulation of what can and cannot be genetically accessed at a certain point of the cell maturation, and therefore contributing together with the genetic information for the phenotype of each cell [5].

These so called epigenetic molecular mechanisms may be grouped into three major categories: DNA methylation, histone post-translational modifications and RNA-associated transcriptional silencing (*Figure 1*).

In addition to these, many other epigenetic mechanisms have been studied, such as histone variants, ATP-dependent chromatin remodeling complexes and polycomb/trithorax protein complexes [6, 7].

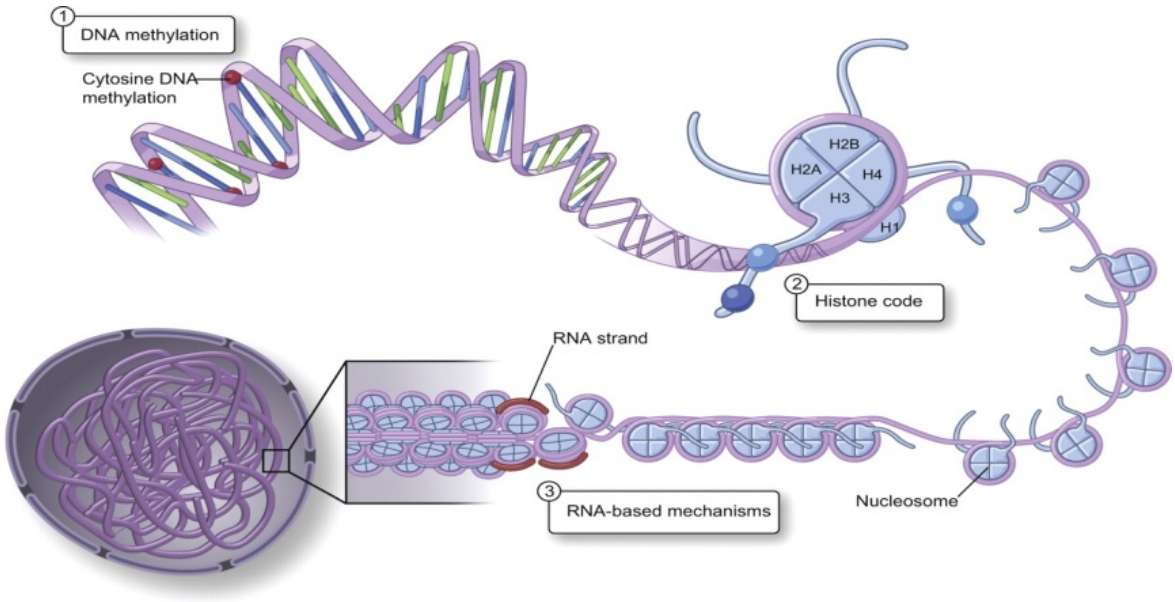

Figure 1. Different types of epigenetic information. DNA methylation, histone modifications, and RNA-mediated gene silencing constitute three distinct mechanisms of epigenetic regulation. (Adapted from [8])

1.1.1. DNA methylation

Cytosine DNA methylation, the most studied of epigenetic changes, is a covalent modification of DNA, in which a methyl group (CH_3) is transferred from S-adenosylmethionine (SAM) to the fifth carbon (C-5) of cytosine by a family of (DNA-5)-methyltransferases (DNMTs), resulting in a new DNA base - 5-methylcytosine (m^5C) (*Figure 2*). This specific DNA base accounts for ~1% of all bases, varying slightly among different tissue types, and corresponding to 3-6% of all cytosines [9] and 70-80% of all CpG dinucleotides [10]. CpG sequences are underrepresented in the genome owing to the evolutionary trend for depletion of such dinucleotides due to spontaneous deamination of m^5C into thymine (T) and the failure of recognition by the mismatch repair mechanism [11-13].

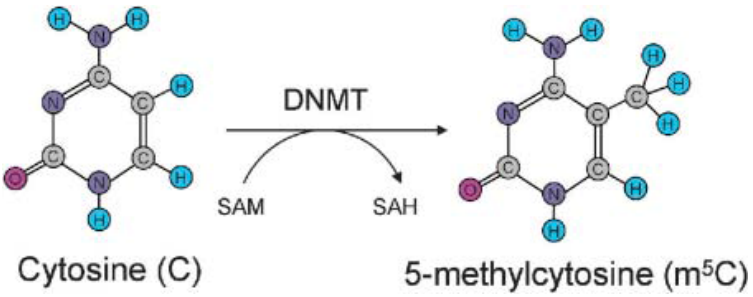

Figure 2. Reaction catalyzed by DNMTs. Addition of a methyl group to the cytosine ring resulting in a 5-methylcytosine using SAM as the methyl donor. (Adapted from [14])

DNA methylation occurs almost exclusively at CpG dinucleotides and has an important role in the gene expression regulation and in the silencing of repeat elements in the genome [15]. Across the genome we often find clustered regions of CpG dinucleotides, also known as CpG islands, defined as regions of more than 200 bases with a G+C content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6 [16]. It is estimated that approximately 60% of human gene promoters contain CpG islands [11] and are normally unmethylated in the majority of cells, corresponding to the maintenance of an open chromatin structure and a potentially active state of transcription [17]. However, some conditions require a change in the gene-promoter unmethylated to methylated status, such as in some imprinted genes, X-chromosome genes in the female gender [18] and in tissue and germline specific genes [19].

In human cells, three major DNMTs are responsible for DNA methylation. Methylation patterns are maintained by DNMT1, which preferentially methylates hemi-methylated DNA (e.g., duplex DNA in which only one of the strands is methylated) following DNA replication. DNMT3A and DNMT3B are known as the *de novo* methyltransferases. Although DNMT3A and DNMT3B show no preference for unmethylated DNA over hemi-methylated DNA, the low level of the *de novo* methylation carried out by DNMT1 relative to DNMT3A and DNMT3B have led to the later two enzymes being designated as the *de novo* methyltransferases [20, 21]. The DNA methyltransferases family includes another two members: DNMT2 and DNMT3L. DNMT2 is the smallest mammalian DNA methyltransferase and it shows reduced methyltransferase activity [20, 22]. DNMT3L is a DNMT-related protein, which does not have a methyltransferase activity. Instead, it interacts with de novo DNA methyltransferases, and appears to have an important role in

imprinting, namely in maternal imprinted genes [6, 9, 20]. Changes in the expression of the DNMTs are a contributing factor to changes in methylation patterns.

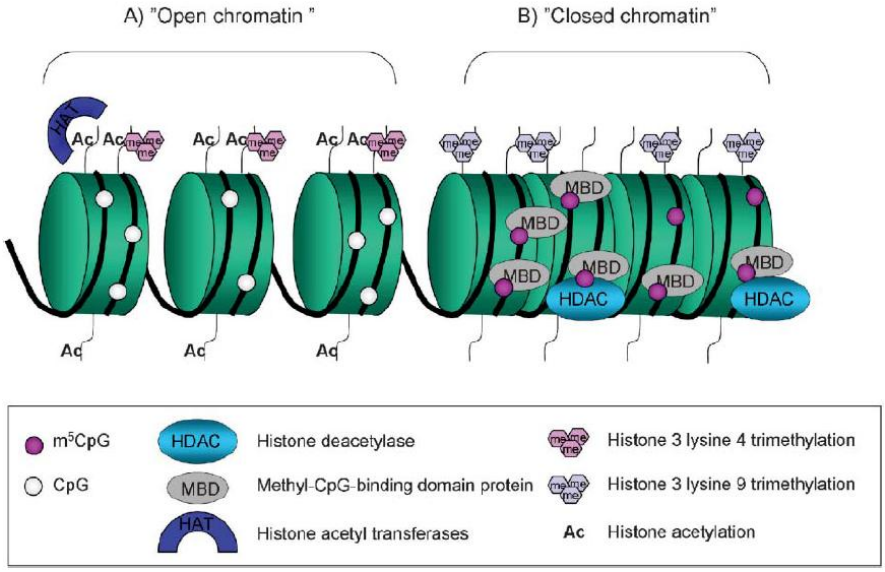

Figure 3. Chromatin structure of active and inactive promoters. A) Transcriptionally active chromatin is characterized by unmethylated cytosines and acetylated histone tails. B) Transcriptionally silenced chromatin is characterized by methylated cytosines and desacetylated histone tails. (Adapted from [14])

As previously mentioned, DNA methylation interferes directly with gene expression by obstructing the action of transcription activators on methylated regions in or near the promoter (*Figure 3A*). In addition to this, another mechanism by which DNA methylation can indirectly regulate transcription is through the recruitment of DNA methyl-binding proteins containing methyl-CpG-binding domains (MBD). These regions contained in proteins such as MeCP1 and MeCP2 include amino acid residues capable of binding to methylated DNA. There are currently several proteins that have been identified and that are referred to as MBD proteins (MBPs), which have the capacity to silence transcription by binding to both hemi-methylated and fully methylated DNA [20, 23]. They act towards the recruitment of transcriptional co-repressors such as histone deacetylating complexes, polycomb proteins, and chromatin remodeling complexes, and attract chromodomain-binding proteins (*Figure 3B*).

Herein, two of the most studied examples of the importance of natural CpG island methylation for the normal development of mammalian cells are the inactivation of one of the X chromosomes in females and the imprinted genes. The first is responsible for the maintenance of stable long-term random

transcriptional silencing of one of the X chromosomes in the female gender, leading to gene-dosage compensation [6, 24, 25]; whereas the second is a non-Mendelian inheritance phenomenon [26], by which one of the parental alleles of a gene becomes transcriptionally silenced, in male or female germlines, leading to monoallelic expression in progeny in a parent-of-origin specific manner [6, 26].

The spontaneous and natural occurrence of DNA methylation not only occurs in the course of cell early development, but also is an integral part of aging and cellular senescence. It is known that the overall content of cytosine methylation decreases with age, while other genes acquire methylation at their promoters, a phenomenon which resembles the methylation changes that are found in cancer [27].

DNA methylation plays an important role in the maintenance of genome integrity by transcriptional silencing of repetitive genomic regions, such as those of pericentromeric regions, transposons (DNA sequences that are able to move across the genome), and inserted viral sequences [28]. In fact, these regions are naturally densely methylated, which in this case comprises a protective effect against chromosomal instability and potentially deleterious recombination events between non-allelic repeats caused by these mobile genetic elements [29]. In addition, as previously stated, methylation increases the mutation rate of m^5C to T, leading to a faster divergence of identical sequences and disabling of many retrotransposons [6].

1.1.2. Histone Post-Translational Modifications

Nucleosomes are the basic units that compose the chromatin fiber. In eukaryotes, each of these primary building units consists of four core histones (H3, H4, H2A and H2B), which form a histone octamer, assembled from two heterodimers of H3 and H4 with two heterodimers of H2A and H2B. Wrapped to the histone octamer are 146 base pairs of DNA, thereby forming the complete structure of the nucleosome. Another histone, H1, binds to DNA between nucleosomes, and this structure is twisted and folded in highly ordered and compacted chromatic filaments [30]. Not so long ago, histones were thought to be static elements, which only function comprised the DNA packing; however, these proteins are currently considered key players in epigenetics, as explained below.

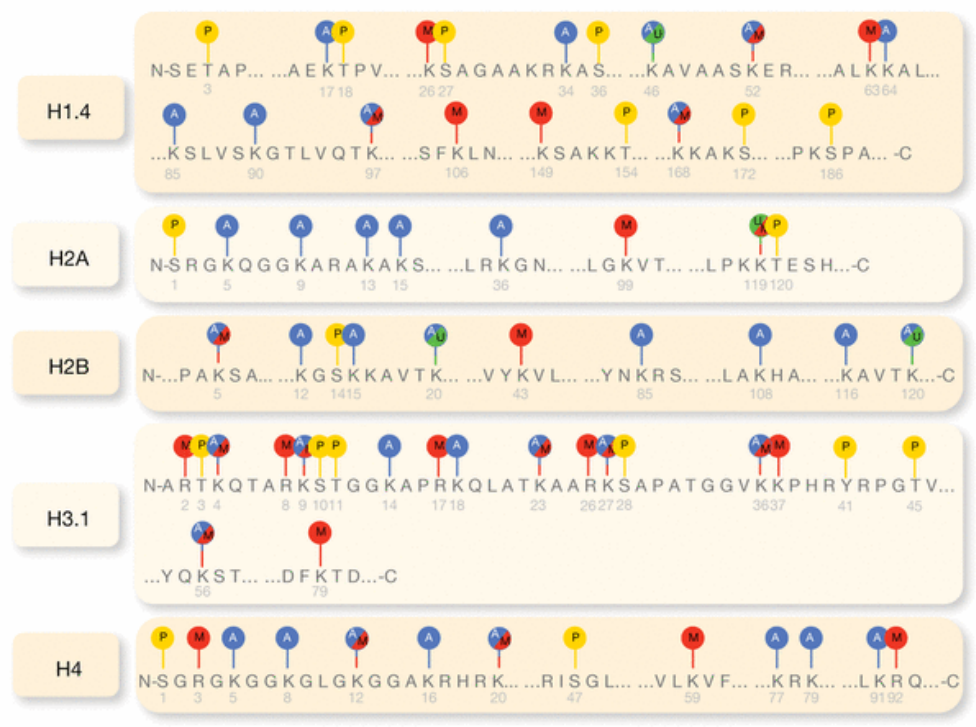

Figure 4. Most frequent post-translational modifications in human histone "tails": A - acetylation; M - methylation; P - phosporylation; U - ubiquitination. (Adapted from [31])

The histone proteins contain a globular C-terminal domain and an unstructured N-terminal tail [32], being the last a target to various residue-specific post-translational covalent modifications, such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and ADP ribosylation (*Figure 4*) [33]. These histone modifications have important roles in various cellular mechanisms, as in transcriptional regulation, DNA repair [34], DNA replication, alternative splicing [35] and chromosome condensation [33], by unwrapping nucleosomal DNA or sliding nucleosomes along it, allowing the protein machinery to access the DNA sequence.

The concept of the "histone code" arises by the establishment of different combinatorial patterns of post-translational modifications in different residues of each one of the nucleosome core histones. Once read out by other proteins, this code is responsible by determining the structure and activity of different chromatin regions, being more or less stable accordingly to the cell environment stimuli [36-38].

The acetylation and methylation status of specific lysine residues contained within the tails of nucleosomal core histones is known to play a critical role in regulating chromatin structure and gene expression.

Lysine acetylation is a reaction catalyzed by histone acetyltransferases (HATs), and is commonly associated with transcriptional activation, whereas the reverse reaction, performed by histone deacetylases (HDACs), leads to a negative gene expression regulation [33]. The above-mentioned transcriptional activation due to acetylation happens because it partially neutralizes the positive charge of histones, weakening their interaction with the nucleosomal DNA, thereby facilitating the access of transcription factors to their recognition element. Additionally, acetylation helps gene transcription by creating a specific signal for regulatory factors or chromatin-remodeling complexes, contributing to their targeting to a specific region [39, 40]. The dynamic equilibrium between HATs and HDACs regulates the overall histone acetylation status [41].

Methylation as a histone post-translational modification is substantially different from acetylation: it may occur at lysines and arginines; histone lysines can become mono-, di-, or trimethylated, whereas arginines may only be mono- or dimethylated (symmetrically or asymmetrically); it does not alter the histone tails charge; and it has been implicated in both transcriptional activation and repression, depending on the altered residue, its position, and its histone code context [40, 41]. For example, open chromatin is characterized by trimethylation of lysines 4, 36 and 79 of H3 (H3K4me3, H3K36me3 and H3K79me3), while heterochromatin features high monomethylation levels of lysines 9 and 27 of H3 (H3K9me and H3K27me), and lysine 20 of H4 (H4K20me) [42]. The effectors of these operations are the histone methyltransferases (HMTs), which catalyze the addition of a methyl group to histone residues using SAM as a cofactor, and display higher substrate specificity when comparing with HATs and HDACs [43]. Together with the HMTs, the histone demethylases (HDMs) are the answer of how histone methylation can regulate transcription and enable gene maintenance in an on or off state [40].

Overall, these histone modifiers generally act together in complexes, such as the repressive Polycomb (PcG) and activating Trithorax (TrxG) group complexes, which counterbalance each other in gene regulation [44].

In normal mammalian cells, histone hypoacetylation and hypermethylation are characteristic of DNA sequences that are naturally methylated and repressed

– such as in the previously mentioned inactive X chromosome in females, and silenced imprinted and tissue-specific genes [45].

1.1.3. MicroRNAs

MicroRNAs (miRNAs) are an evolutionary conserved group of small RNAs (18-24 nucleotides) that are processed from much longer transcripts, by RNA polymerase III, and arise from hairpin structures after successive enzymatic maturation steps, performed by the sequential action of Drosha and Dicer endonucleases [46]. Following incorporation into the ribonucleoprotein complex RISC (RNA-induced silencing complex), the miRNAs bind mRNAs, primarily at their 3' untranslated regions (UTRs), through partial complementarity of their both sequences. Consequently, mRNA suffers decay and the translation is suppressed, leading to a reduction on protein levels. Moreover, recent reports showed that some miRNAs might also bind to the 5' UTR of the target genes, functioning as transcription activators [46-48].

The behavior of this class of molecules is considered somehow promiscuous, because each miRNA has many targets and the individual mRNAs can be targeted by multiple miRNAs [49]. Actually, nowadays miRNAs are considered one of the most important groups of gene regulatory molecules, by controlling a wide range of biological processes, such as differentiation, proliferation, and apoptosis. A connection between epigenetics and miRNAs has been sustained by the recent identification of the "epi-miRNAs" specific subgroup, which can directly and indirectly modulates the activity of the epigenetic machinery [50].

2. Cancer: a Global Threat

Cancer has been the main concern of many life sciences researchers and medical doctors worldwide, as it affects all kind of people of all ages and social status. The term *Cancer* was first described by Hippocrates, in the ancient Greece, and the oldest known description and surgical treatment of cancer was discovered in Egypt and dates back to approximately 1600 BC. Cancer is not one disease but rather a large and heterogeneous group of diseases that share similar attributes: the cells show uncontrolled growth as a result of mutations that affect a limited number of genes. In the past century, with the discovery of the main molecular mechanisms of the cells, we now know that the carcinogenic process confers selective growth advantages (*e.g.*, evasion of apoptosis and metastasis formation) to the tumor, and gives rise to its proliferation, invasion, dissemination, and drug resistance traits [51].

Most cancers are collections of phenotypically mixed cell populations with variable proliferative potential, a feature shared with normally developing organs. Two primary models have gained support to account for this variability. The clonal evolution model proposes that multiple genetic mutations confer upon a cell the ability to proliferate indefinitely, and that a subsequent mutation will confer a growth advantage upon one of this cell's progeny. The new, more rapidly growing clone will outcompete other clones and dominate the tumor [52, 53]. The cancer stem cell model provides an alternative explanation, assigning cellular heterogeneity to differing degrees of differentiation. According to this model, heterogeneity is predictable rather than random, recapitulating aspects of the developmental and homeostatic mechanisms of normal tissues.

2.1. Epigenetic Modifications in Cancer

Recent advances in the field of Epigenetics have shown that human cancer cells, in addition to the previously mentioned genetic alterations, harbor global deregulation of epigenetic events (so called "epimutations"). In fact, these epigenetic alterations may work as a trigger of neoplastic development, working together with genetic mutations to promote cancer progression [29]. Cancer is

now seen not only as a genetic based disorder, but also as an epigenetic disease, since epimutations are increasingly associated with the silencing of tumor-suppressor genes (TSGs) and activation of proto-oncogenes [5, 54].

With the progress that has been made in the study of different epigenetic mechanisms, an increased knowledge of how these mechanisms may influence malignant cellular transformation as been gathered, as detailed bellow.

2.1.1. DNA Methylation in Oncogenesis

Cancer initiation and progression is accompanied by deep variations in DNA methylation landscape. Indeed, a cancer cell is characterized by genome-wide loss of m^5C, contrasting with the subsequent hypermethylation of CpG islands, more precisely TSGs CpG island promoter methylation (*Figure 5*) [54, 55].

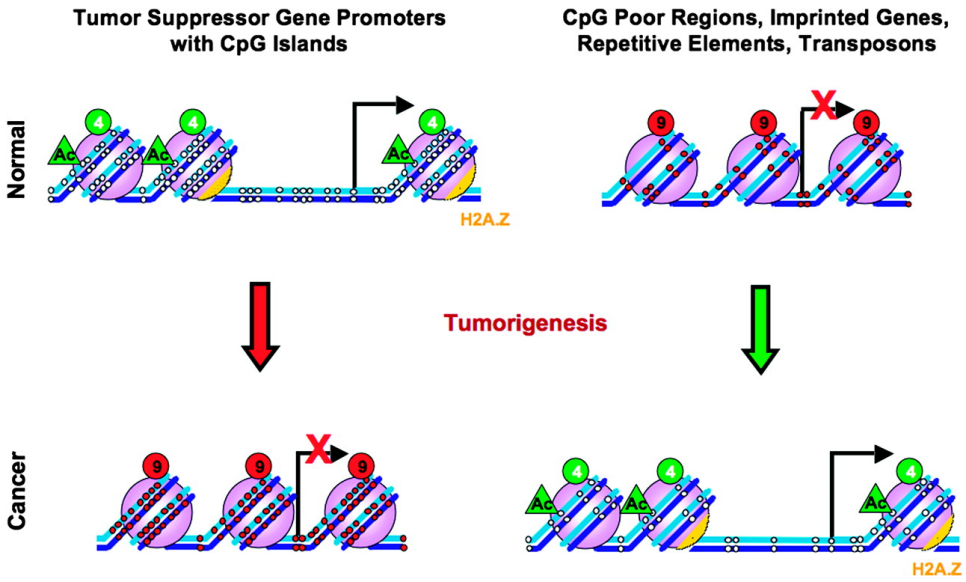

Figure 5. DNA methylation changes in cancer: TSGs promoter hypermethylation and global genome-wide methylation loss. (Adapted from [5])

Global DNA hypomethylation was one of the first epigenetic alterations reported in cancer cells, more than three decades ago (Feinberg and Vogelstein, 1983). In fact, it is known that a cancer cell features a loss of 20-60% of the total number of m^5C when compared to a normal cell from the same tissue [13]. Moreover, it has been clearly established that hypomethylation occurs early in tumorigenesis and it is a feature of different types of human neoplasms (benign or malignant), increasing with disease progression [2, 56].

This epigenetic change is mainly due to demethylation of repetitive DNA sequences, coding regions, and introns of genes. The consequences of the methylation loss of this particular sequences may represent: loss of imprinting [57]; generation of chromosomal instability, because demethylation of DNA can favor mitotic recombination, leading to deletions and translocations [58, 59]; re-activation of transposons [60, 61]; and activation of normally methylated oncogenes [62]. Moreover, there are still several loose ends in the research regarding the effects of hypomethylation in cancer.

On the other hand, the effects of hypermethylation in tumorigenesis are much better established and vastly studied, and there is strong evidence that this epigenetic alteration affects both malignant initiation and progression. Indeed, considering Knudson's two-hit hypothesis for inactivation of TSG [63], in addition to a mutation or other genetic phenomena in one of the alleles, the other necessary event may well be the promoter aberrant methylation, therefore activating the tumorigenic mechanisms [64]. Alternatively, both alleles of a TSG may also become inactivated by this epigenetic event.

Actually, it is acknowledged that the transcriptional inactivation caused by promoter hypermethylation affects genes involved in the main cellular pathways, such as in the promoters of *MGMT* and *BRCA1* (involved in DNA repair), *RB* (cell cycle control), and *WIF-1* and *DAPK1* (apoptosis), among others [65]. The list of genes undergoing silencing through methylation in several different types of cancer is now very extensive, as the research in this particular field is increasingly sought.

2.1.2. Histone onco-modifications

While aberrant DNA methylation is the most extensively studied epigenetic change in cancer, recent discoveries have revealed that both deregulation of histone modifications and chromatin remodeling are also implicated in cancer. Moreover, it has become evident that DNA methylation and histone modification pathways can be dependent on one another, and that this cross-talk can be mediated by biochemical interactions between HMTs and DNMTs [66].

If taken into consideration the previously discussed fundamental roles of histone modifications, it is not surprising that aberrations in histone modifications are discovered in cancer. These alterations may lead to mutations in oncogenes, TSGs or DNA repair genes resulting in genomic instability,

oncogenic transformation and the development of cancer [5]. Thus far, only a few of the total number of histones residues in which modifications have been described were linked to malignant transformation [33]. It is the example of global loss of acetylated H4K16, the trimethylation of H4K20, and the changes in methylation patterns of H3K9 and H3K27, found in various forms of cancer [67].

In addition, several of the histone-modifying enzymes have also been described to be altered in cancer cells [56]. HDACs are frequently found overexpressed in several types of cancer, and HATs, which work together with HDACs to preserve histone acetylation levels, may also be altered in those situations [68]. Similarly, deregulation of HMTs results in altered distribution of histone methylation marks in cancer, such as EZH2 (the H3K27 HMT) that is often found overexpressed in breast and prostate cancer [69].

Additionally, it is currently known that the incorporation of specific histone variants is altered in cancer cells. In fact, various researchers are focusing their efforts in understanding how this alternative histones influence tumorigenesis, as in the example of a 2011 study in which the authors showed that during sequential development of hepatocellular carcinoma, the histone variants H2A and H2A.1 were overexpressed, whereas H2A.2 had decreased [70].

2.1.3. MicroRNA Deregulation in Cancer

Firstly identified in B-cell chronic lymphocytic leukaemia (CLL) [71], changes in the expression levels of miRNAs have been successively found by different authors in many types of human neoplasms.

MiRNAs have been proposed to contribute to oncogenesis since their function implies regulation of genes involved in transcriptional regulation, cell proliferation and apoptosis, and aberrant expression of those miRNAs favors tumorigenesis. Importantly, miRNAs can function either as tumour suppressors (as is the case for miR-15a and miR-16-1) or oncogenes (as is the case for miR-155 or members of the miR-17–92 cluster), depending upon their target genes [72].

This miRNAs expression deregulation may occur as a consequence of amplification, deletion, mutation, chromosomal abnormalities, changes in expression of transcription factors and gene promoter methylation [73, 74]. Indeed, those miRNAs with tumor suppressor features could undergo aberrant DNA methylation accompanied by histone modifications associated with

transcriptional inactivation. Several studies have been made to support this hypothesis, namely a recent report showed that breast cancer cells treated with histone deacetylase inhibitors (HDACi) display an extensive and rapid alteration of miRNA levels [75].

2.2. Epigenetic Therapy

The dynamic nature and potential reversibility of DNA and histones modifications renders them as appealing therapeutic targets in cancer intervention. Indeed, the aim of this so called *epigenetic therapy* is to reverse the causal epigenetic abnormalities that arise in cancer, leading to the reinstatement of a normal epigenome landscape [76].

Multiple therapies have been tested over the past decade to treat neoplasia through demethylation of DNA. Most of those therapies reduce methylation through DNMTs inhibitors (DNMTi). One class of compounds handed as demethylation agents are nucleoside analogues. They have a structure similar to that of cytosine and when incorporated into DNA they will covalently bind DNMTs to the DNA. The DNMTs will then remain bound to the DNA preventing them from carrying out methylation elsewhere. Examples of these compounds are azacytidine, zebularine and decitabine. Remarkably, the FDA has approved the use of two such agents, 5- azacytidine and 5-aza-2′-deoxycytidine (DAC), as elective treatments for myelodisplastic syndrome [76]. Other molecules such as Genistein, procainamide, antisense oligonucleotides and siRNAs have all been shown to reduce hypermethylation.

Not only DNMTi have shown promising results in cancer fight front, but also the HDAC inhibitors (HDACi), which have demonstrated antitumor, growth inhibitory, proapoptotic, and prodifferentiation properties [77]. These antiproliferative properties of HDACi are mediated by their ability to reactivate TSGs, and because of that there is intense trial activity regarding these compounds [78]. Actually, suberoylanilide hydroxamic acid was the first drug of this type approved by the FDA for the treatment of cutaneous T-cell lymphoma [76, 79], and more recently romidepsin showed great improvements in the treatment of the same condition, being also approved by FDA in 2009 [80]. Another HDACi, Trichostatin A (TSA), has been tested *in vitro* and it was demonstrated that its activity may lead to apoptosis, as well as differentiation

and cell-cycle arrest [81]. Moreover, several other molecules are being tested in clinical trials.

A schedule-dependent synergistic effect between DAC and TSA has already been tested by various groups, and resulted in to the re-expression of previously methylated promoters, emphasizing the complementarity/interaction between the different epigenetic gene regulation mechanisms [82, 83].

2.3. Cancer Biomarkers: The Epigenetics contribution

There is a great need for novel methods able to detect cancer at an early stage. Evaluation of molecular changes in tumor cells might be a useful ancillary tool for the existing detecting methods in the early detection of various types of cancer, specifically of urological cancers [84].

A cancer biomarker is any biological substance that is indicative of the presence of the tumor in the body. It can be derived from the tumor site, remote media or found in circulation. These biomarkers include cell-free tumor derived DNA, RNA, and protein.

In addition, epigenetic changes, including DNA methylation, have been used to detect cancer. Owing to their chemical and biological stability, DNA methylation-based biomarkers have potential clinical applications in cancer detection, diagnosis, and targeted therapies [85]. As previously mentioned, epigenetic changes, associated with loss of gene expression, are common events in cancer, and hypermethylation is the most studied of all. The finding that DNA methylation is an early occurrence in cancer coupled with the chemical and biological stability of this alteration makes it a potential diagnostic tool for cancer [86].

DNA methylation assays can be performed on small biopsy samples obtained during the routine diagnostic work-up of patients, on archived frozen or paraffin-embedded tissue, as well as on the soluble genomic DNA found in the peripheral blood and other fluids of many cancer patients, offering the possibility of non-invasive molecular screening for many malignancies [87].

The efficacy of a biomarker assay is determined by its sensitivity and specificity. Clinical sensitivity of a biomarker is determined by the ratio of cancer cases that test positive among the total number of cases tested. Specificity is assessed by the number of healthy individuals that test negative for the particular

biomarker. In this process of biomarker selection, it is also important to consider other factors that influence gene methylation. Since DNA methylation aberrations are affected by factors such age, race, diet, and stage of disease, it is important to analyze gene methylation data by taking these variables into consideration [86].

2.4. GDF15, TMEFF2 and VIM

In a previous work of our group [88], a panel of three epigenetic biomarkers – *GDF15, TMEFF2* and *VIM* – was identified for accurate identification of urothelial carcinoma of the bladder.

The *GDF15* gene, located at 19p13.11, encodes a divergent member of the transforming growth factor (TGF)-β superfamily, which is a large family of secreted molecules required for normal development, differentiation, and tissue homeostasis. It has been postulated that, like other members of the TGF-β superfamily, *GDF15* might act as tumor suppressor in early cancer stages and as a pro-tumorigenic at later stages of tumor progression [89].

The *TMEFF2* gene, located at 2q32.3, encodes a transmembrane protein with EGF-like domains and two follistatin-like domains, involved in cell proliferation control. The *VIM* gene, located at 10p13, encodes the intermediate filament Vimentin. Both *TMEFF2* and *VIM* had previously found to be silenced through aberrant promoter methylation in esophageal, gastric and colon cancer [90-94].

3. Upper Urinary Tract Urothelial Cell Carcinoma

3.1. Upper Urinary Tract: Anatomical Insights

The human urinary tract may be distinguished between upper and lower segments. The upper tract includes the renal pelvis and the ureters, and the lower tract comprises the bladder and urethra. Specifically, the ureters are hollow, muscular tubes that carry urine from the kidneys to the bladder, and the renal pelvis is the lower part of each kidney that connects to each ureter (*Figure 6*).

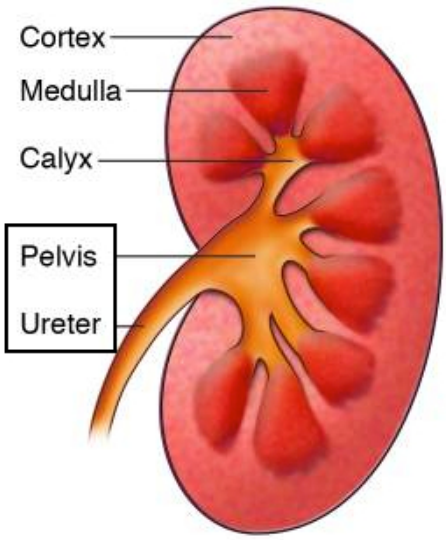

Figure 6. Anatomy of the human upper urinary tract. (Adapted from [95])

Urothelium, from which all the urothelial tumors originate, is a highly specialized epithelium, which covers most of the urinary tract. Situated strategically between the urine and blood, normal urothelium acts as a physiologically effective and mechanically flexible permeability barrier that on the one hand protects the underlying tissues from toxic urinary substances and on the other hand adjusts its surface area actively and reversibly during the micturition cycle [96].

3.2. Epidemiology of Upper Tract Urothelial Cancer

Urothelial cell carcinomas (also known as transitional cell carcinomas) are the fourth most common tumors worldwide after prostate (or breast), lung

cancer, and colorectal cancer [97, 98]. However, Upper Tract Urothelial Carcinomas (UTUCs) are uncommon and account for about 7% of renal neoplasms, and only 5 to 10% of all urothelial cell carcinomas. Moreover, the estimated annual incidence of UTUCs in Western countries is about two new cases per 100 000 inhabitants [97, 99]. However, despite presenting more rarely, most UTUCs (60%) are invasive at diagnosis, in contrast to the 15-25% in bladder urothelial carcinoma [100].

These tumors have a peak incidence in the seventh decade, and they are three times more prevalent in adult males than in females [101]. However, as is the case with bladder cancer (BlCa), women who develop UTUC are 25% more likely than men to die of their disease [99, 102].

3.3. Classification and Staging

In the past few years, with the advance in the knowledge of the molecular basis of UTUCs, the World Health Organization (WHO) changed its classification based on a grade scale to a more suitable classification that better show the natural evolution of these tumors, and takes into consideration histological insights. Moreover, these tumors are usually classified as low-grade papillary urothelial carcinoma, high-grade papillary urothelial carcinoma and high-grade invasive urothelial carcinoma, according to 2004 WHO classification. In addition, UTUC also follow the TNM stage classification of malignant tumors, which assess tumor size, lymph node involvement, and distant metastasis (*Table 1*) [103].

Table 1. TNM classification of carcinomas of the renal pelvis and ureter. (Adapted from [103]

TNM classification[1,2]

T – Primary tumour

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ
T1	Tumour invades subepithelial connective tissue
T2	Tumour invades muscularis
T3	*(Renal pelvis)* Tumour invades beyond muscularis into peripelvic fat or renal parenchyma *(Ureter)* Tumour invades beyond muscularis into periureteric fat
T4	Tumour invades adjacent organs or through the kidney into perinephric fat

N – Regional lymph nodes

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node 2 cm or less in greatest dimension
N2	Metastasis in a single lymph node more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension
N3	Metastasis in a lymph node more than 5 cm in greatest dimension

M – Distant metastasis

MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage Grouping

Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
Stage IV	T4	N0	M0
	Any T	N1, N2, N3	M0
	Any T	Any N	M1

[1] {944,2662}.
[2] A help desk for specific questions about the TNM classification is available at http://www.uicc.org/tnm/

3.4. Risk Factors

Presently, there are several environmental risk factors that are known to influence the development of UTUCs, and the great majority of them are shared with BlCa, with few exceptions. Among them, tobacco smoke, occupational exposure to aromatic amines, and the Balcan endemic nephropathy remain the best established, as explained bellow [104].

The association of urothelial carcinogenesis with smoking exposure is complex and linked to multiple inhaled toxic substances, as the example of aromatic amines. The mechanism of action includes its metabolization into N-hydroxyalanine, which is proven to have a real carcinogenic activity. The relative risk (RR) of developing UTUC under tobacco exposure increases 2.5–7, comparative with non-smokers [105]. This risk is modulated by the number of years of exposure and by the number of cigarettes smoked every day.

Occupational exposure to aromatic amines can also lead to the development of UTUC. These aromatic hydrocarbons are used in many industries (eg, dyes, textiles, rubber, chemicals, petrochemicals, and coal). Benzidine and b-naphthalene are two known chemicals that belong to this family, and have a documented carcinogenic potential. Precisely because of that, these two chemicals have been banned since the 1960s in most industrialized countries. Yet about occupational exposure, researchers found that the developing of UTUC only occurs after an average duration of exposure of approximately seven years, and that the estimated risk (odds ratio) of developing UC after exposure to aromatic amines is 8.3 [104, 106].

Another well-known risk factor for the developing of UTUC is the Balcan endemic nephropathy (BEN). BEN is described as a proximal tubular dysfunction responsible for the presence of a low molecular weight protein and a dense interstitial fibrosis within the *glomeruli*, and is intrinsically associated with UTUC, in the rural areas of the Balkans. The evidences indicate that this association is due to the consumption of chinese herbal products containing *Aristolochia fangchi* (a plant endemic to the Balkans) [107, 108]. Its mechanism of actions causes a specific mutation in the *p53* gene at codon 139. This mutation is very rare in the non-exposed population and predominant in patients with nephropathy due to Chinese herbs or BEN that present with UTUC.

3.5. UTUC Diagnosis and Prognosis Assessment

3.5.1. Diagnosis and Treatment

The type and severity of clinical signs and symptoms of UTUCs depends on the extent and location of the tumor. The most common first presenting symptom is microscopic hematuria, although most tumors are clinically silent until they reach advanced disease stages.

Nowadays, the diagnostic approach for UTUC is through CT-urography, cystoscopy and urinary cytology, and the gold standard treatment for those tumors is open radical nephroureterectomy (RNU) [99]. Endoscopic management, however, is also a reasonable therapeutic option in selected patients (*Figure 7*). Some new diagnostic approaches have been tested in the past few years, such as the use of nomograms [109] and fluorescence in situ *hybridization* [110].

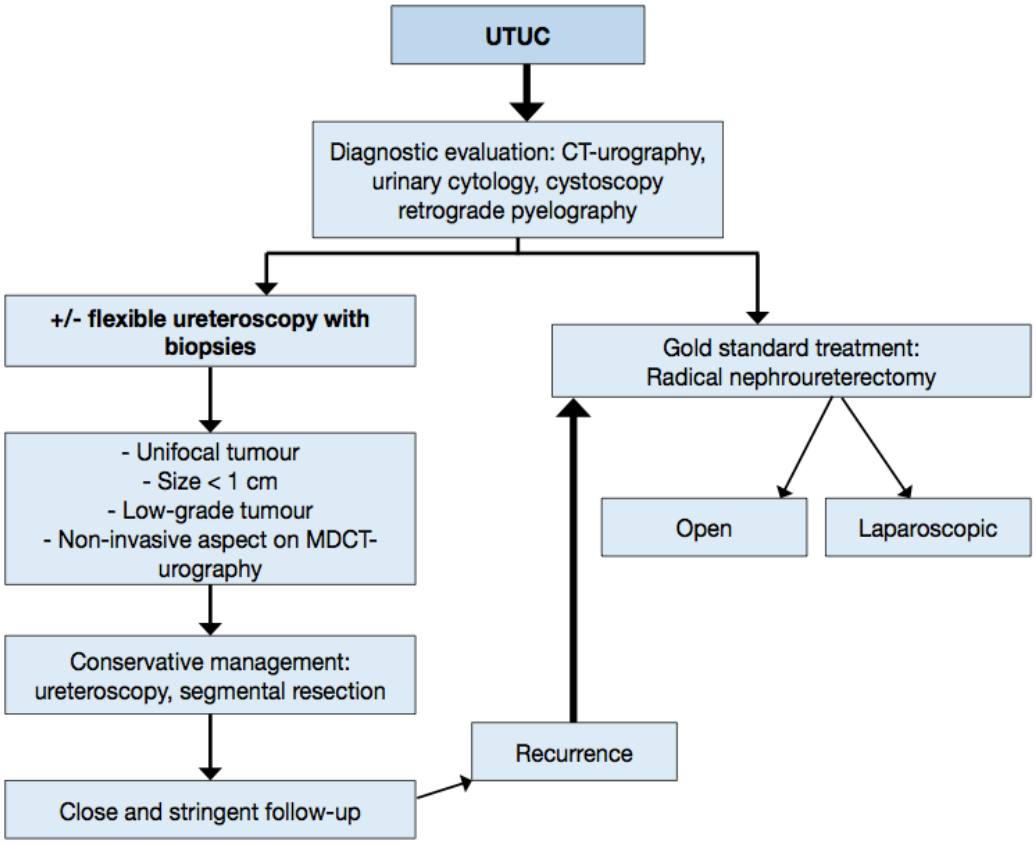

Figure 7. Proposed flowchart for the management of UTUC. (Adapted from [99])

Because of the current invasive diagnostic and treatment techniques, UTUCs are optimal candidates for the previous mentioned *biomarkers* approach, as it would aid in their early detection and assessment of prognosis.

3.5.2. Prognostic Factors

There are several factors that determine the decisions made by clinicians in diagnose and management of UTUC.

As expected, the more important prognostic factors for UTUC progression are the tumor stage and grade, as well as the location of the primary tumor within the upper tract (eg, ureter *vs*. renal pelvis), being the ureteral tumors those with worst outcome [111, 112]. Other factors such as tumor necrosis, surgical margins of the resected tumor, and lymphovascular invasion, are also taken into consideration.

Additionally, patients' age is also taken into consideration, because older age at the time of RNU is associated with decreased cancer-specific survival [113]. Nonetheless, when the addition of age in a model that includes standard pathological features for prediction of disease recurrence did not alter or improve the predictive accuracy of the same, it was established that RNU should not be denied to elderly patients [114].

3.5.3. Molecular Markers

The most studied factors for assessing the biomarker potential of UTUCs are proteins involved in phenomena such as cell adhesion, angiogenesis, cell proliferation, apoptosis and cell-cycle regulation. *Table 2* shows a review of the most studied UTUC markers until today.

It has been reported that the expression profiles of E- cadherin and N-cadherin, particularly the gain of N-cadherin expression, were significantly associated with disease recurrence after RNU in UTUC [115]. It can be used in a group of patients that require a thorough follow-up. Also, the expression of hypoxia-inducible factor 1α, a protein involved in angiogenesis, more specifically in cell hypoxia adaptation, has shown association with UTUC recurrence and overall mortality. Moreover, it was associated with grade, pattern of growth, but not with stage, and it was undetectable in normal urothelium [116]. Similarly, Ki-67 protein, which is a known marker of cell proliferation, has been found to correlate with tumor grade and stage [117]. In fact, several studies suggest that high Ki-67 index is indicative of a worse prognosis.

Table 2. Summary of the main markers of interest in UTUC. (Adapted from [113])

Markers	Function	Detection	Comment
Tissue-based			
p53	Cell-cycle regulation	Immunohistochemistry	Overexpression is associated with advanced T stage and higher tumor grade.
Ki-67	Cell proliferation	Immunohistochemistry	Overexpression is associated with advanced T stage and higher tumor grade. It is an independent predictor of synchronous/ metachronous bladder cancer.
EGFR	Cell proliferation and differentiation	Immunohistochemistry	Overexpression is associated with advanced disease and metaplastic differentiation.
Uroplakin III	Cell differentiation	Immunohistochemistry	Loss of expression is associated with advanced disease. It is an independent predictor of lower CSS rates.
Snail	Cell differentiation	Immunohistochemistry	Overexpression is associated with advanced T stage and higher tumor grade. It is an independent predictor of lower RFS and CSS rates.
Bcl-2	Apoptosis	Immunohistochemistry	Overexpression is associated with advanced T stage and higher tumor grade.
Survivin	Apoptosis	Immunohistochemistry	Overexpression is associated with advanced T stage and higher tumor grade. It is an independent predictor of lower CSS rates.
Telomerase mRNA	Maintaining DNA integrity	In situ hybridization	Overexpression is an independent marker for lower FRS and OS rates.
HIF-1α	Angiogenesis	Immunohistochemistry	Overexpression is an independent marker for lower RFS and OS rates.
Metalloproteinases	Angiogenesis	Immunohistochemistry	Overexpression is associated with advanced T stage. It is an independent predictor of lower CSS rates.
E-cadherin	Cell adhesion	Immunohistochemistry	Lower levels are associated with advanced disease and are an independent predictor of lower progression-free survival, RFS, and CSS rates.
β-Catenin	Cell adhesion	Immunohistochemistry	Loss of normal expression is an independent marker for lower progression-free survival and CSS rates.
Parvin-β	Cell adhesion	Immunohistochemistry	Lower expression is an independent marker for lower CSS rates.
CD24	Cell adhesion	Immunohistochemistry	Expression is associated with advanced T stage and higher grade.
Blood-based			
C-reactive protein	Inflammatory response	ELISA	Elevated levels are independently associated with lower RFS and CSS rates.
Leukocytes	Inflammatory response	Cytometry	Elevated levels are independently associated with lower RFS and CSS rates.
Alkaline phosphatase	Hydrolase enzyme	Spectrophotometry	Elevated levels are independently associated with lower RFS and CSS rates.
CYFRA 21-1	Cell structural integrity	ELISA	Elevated levels are independently associated with lower OS rates.
Genetic			
Microsatellite instability	Defect in DNA repair process	PCR	Microsatellite instability is an independent marker for lower CSS rates.
Promoter hypermethylation	Repression of gene transcription	PCR	Promoter hypermethylation is associated with advanced disease and is an independent marker for lower progression-free survival rate
FGFR-3	Cell proliferation and differentiation	PCR	Mutations in the *FGFR* gene are associated with milder disease and better survival.
Urine			
Chromosomal alterations	Defect in DNA repair process	FISH	Chromosomal alterations increase sensitivity and specificity for UTUC detection.

Interestingly, UTUC tumors with metaplastic differentiation have been demonstrated to be more resistant to chemotherapy. Recent studies suggested that the epidermal growth factor receptor (EGFR) expression is associated with

advanced disease and metaplastic differentiation, having a potentially significant prognostic value in this particular type of tumor [118]. Recently, in a series of 82 UTUC cases, the tumors overexpressing EGFR were of high-grade and this event may be of interest for targeted therapy [119].

Telomerase has been found to be reactivated in most human cancer tissue but repressed in normal somatic tissues. To investigate the use of telomerase as a marker in UTUC, telomerase RNA component (hTR) overexpression detected by in situ hybridization has been identified as an independent prognostic marker of both UTUC recurrence and survival. Moreover, hTR was suggested for UTUC diagnosis, as it was detected in most cases of these tumors [120].

UTUC has also been associated with tumors exhibiting microsatellite instability, a phenomenon where microsatellite regions of DNA get longer or shorter (e.g. expansion or deletion) owing to mutations in genes that repair damaged DNA. These alterations can be easily detected in exfoliated urine by PCR amplification with DNA from tumor and normal tissues, and has good overall sensitivity and specificity. Cases of UTUC have been linked to hereditary non-polyposis colon cancer (HNPCC), a condition caused by a germline mutation in a mismatch repair gene, and with high MSI levels. Therefore, UTUC tumors with high MSI levels are classified as hereditary, and associated with HNPCC [121, 122]. In addition, MSI can be also detected in ~25% of sporadic UTUC cases, and functions as an independent prognostic factor that could identify a subset of patients with a better prognosis [123].

Moreover, back to the epigenetics, and knowing that a great percentage of UTUC has MSI, Catto and his colleagues observed that methylation can be the responsible for hMLH1 inactivation in those cases, and was associated with advanced tumor stage, suggesting that this group of tumors might benefit from the development of novel therapies with demethylating agents [124].

Due to the rarity of this disease, although there have been various studies investigating the diagnostic, and mainly, the prognostic value of several molecular biomarkers for patients with UTUC, most of them are single center, small in size and lack external validation.

AIMS OF THE STUDY

This Master Thesis project was performed within the Cancer Epigenetics Group of the Research Center of the Portuguese Oncology Institute – Porto. In a previous work of our research team, a panel of three epigenetic biomarkers – promoter methylation of *GDF15*, *TMEFF2* and *VIM* – was discovered to accurately identify bladder UC. Indeed, a 100% sensitivity and specificity was found in tissue samples, and 94% sensitivity and 100% specificity in urine samples [88]. Because bladder UC and UTUC display clinical and genomic similarities [125], we hypothesized that the same panel of biomarkers might be useful for non-invasive, early detection of UTUC. Therefore, we used these previous findings to design the aims of this new project, which comes to live in this Thesis.

Thus, the major aims of this study were:

- ⇒ Evaluate the performance of quantitative *GDF15*, *TMEFF2* and *VIM* promoter methylation in a consecutive series of tissue samples from UTUC;
- ⇒ Determine the feasibility of detecting UTUC in voided urine samples suspicious of malignancy;
- ⇒ Assess whether the panel of genes has prognostic value for this type of cancer;
- ⇒ Explore the biological significance of *VIM* gene in the pathways of UTUC carcinogenesis using transitional carcinoma cell lines.

MATERIAL AND METHODS

1. Clinical Samples

1.1. Patients and tumor sample collection

Fifty-seven UTUC samples were obtained from a consecutive series of patients diagnosed and treated with RNU or ureterectomy at the Portuguese Oncology Institute – Porto, Portugal, between 2000 and 2011, with no previous history of bladder cancer. Tissues were routinely fixed and paraffin-embedded for standard pathologic examination, allowing for tumor classification and WHO/ISUP grading [103], and staging [126]. Additionally, an independent set of 36 paraffin-embedded normal upper tract urothelium (NUTU) from renal cell carcinoma patients was used as controls. Relevant clinical data was collected from clinical charts, and can be consulted in *Table 3*.

Table 3 - Clinical and histopathological parameters of patients with UTUC and NUTU.

Clinicopathological features	UTUC	NUTU
Patients, n	57	36
Gender, n (%)		
Male	39 (68)	23 (64)
Female	18 (32)	13 (36)
Median age, yrs (range)	72 (52 - 85)	62 (49 - 82)
Pathological stage, n (%)		
pTa	4 (7)	*n.a.*
pT1	22 (38)	*n.a.*
pT2	13 (23)	*n.a.*
pT3	17 (30)	*n.a.*
pT4	1 (2)	*n.a.*
Grade, n (%)		
Papillary, low-grade	15 (26)	*n.a.*
Papillary, high-grade	23 (41)	*n.a.*
Invasive, high-grade	19 (33)	*n.a.*

1.2. Urine sample collection and processing

Voided urine samples (one per patient) were collected from 22 patients with UTUC who were diagnosed and treated between 2006 and 2012 at Portuguese Oncology Institute - Porto, Portugal.

A set of 20 voided urine samples from patients with renal cell carcinoma (n = 10), prostate carcinoma (n = 7), and healthy blood donors with no personal or family history of cancer (n = 3), were also collected and used for control purposes (*Table 4*). Informed consent was obtained for urine sample collection of patients and controls (IRB-CES-IPOFG-EPE 019/08).

Urine storage and processing conditions were standardized: each sample was immediately centrifuged at 4000 rpm for 10 minutes, the pelleted sediment was then washed twice with phosphate-buffered saline (PBS), and stored at -80ºC.

Table 4 - Clinical and histopathological parameters of patients with UTUC and gender and age distribution of the control set individuals, who provided urine samples for this study.

Clinicopathological features	UTUC	Control Set
Patients, n	22	20
Gender, n (%)		
Male	12 (54)	11 (55)
Female	10 (46)	9 (45)
Median age, yrs (range)	73 (53 - 86)	61 (48 - 83)
Grade, n (%)		
Papillary, low-grade	3 (14)	*n.a.*
Papillary, high-grade	12 (54)	*n.a.*
Invasive, high-grade	7 (32)	*n.a.*

2. Quantitative Methylation Analysis

2.1. Nucleic acids isolation

From each case, a representative paraffin block was selected and an experienced uropathologist delimited the area of tumor to be macrodissected. Then, a set of 10-20 serial 7-micrometer thick sections were cut and placed on glass slides. A disposable sterile scalpel blade was used to macrodissect the tumor areas which were subsequently placed in labeled 1,5mL microcentrifuge tubes.

Tissue samples were then deparaffinized using Xilol and Ethanol 100%, 90%, 70% and 50%, and digested in 1000μL of digestion buffer, composed by Tris-HCl 1M, EDTA 0,1M, Tween 20 and sterile bidistilled water (B.Braun, Melsungen, Germany), plus proteinase K (20mg/ml, 50μL) (Sigma-Aldrich®, Germany), by incubation for 2 to 3 days in a water-bath at 55ºC, until total digestion was accomplished.

DNA was extracted from tissue and urine samples by the standard phenol-chloroform procedure [127], using 500 μL of phenol-chloroform solution at pH 8 (Sigma-Aldrich®, Germany; Merck, Germany) in Phase Lock Gel Light tubes (5 PRIME, Germany). After centrifuging the tubes for 15 min at 13,000rpm, the upper aqueous phase containing DNA was transferred to a new tube, and then precipitated at -20ºC overnight using chilled Ethanol 100% (2 volumes of original amount of this phase), Ammonium Acetate 7,5M (1/3 volume) (Sigma-Aldrich®, Germany) and glycogen (2μL). This step was followed by two centrifugations at 13,000rpm for 20 min with 70% ethanol, and the pellets were then air dried and eluted in sterile distilled water (B.Braun, Melsungen, Germany).

After DNA elution, its concentrations were determined using ND-1000 Nanodrop (NanoDrop Technologies, Wilmington, USA).

2.2. Sodium Bisulfite Treatment of DNA

The methylation status of a DNA sequence can be determined using sodium bisulfite modification-based protocols. Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged (*Figure 8*).

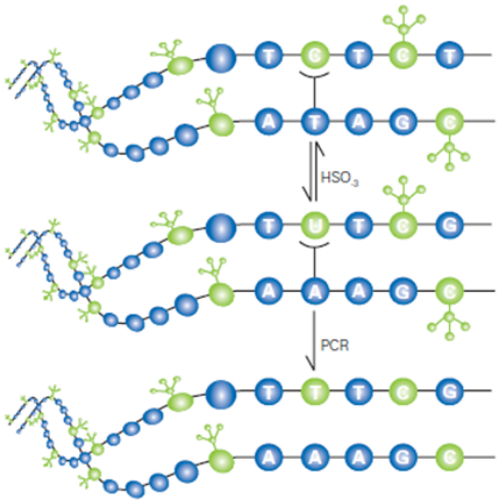

Figure 8. Diagram of bisulfite conversion of unmethylated cytosine, followed by PCR. (Adapted from Workflow guide – Epigenetics and DNA Methylation [Applied Biosystems])

This method transforms an epigenetic event into a genetic change, which is then able to be analyzed using PCR-based methods. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA.

Previously extracted and quantified DNA from clinical samples was treated with the EZ DNA Methylation-GoldTM Kit (Zymo Research, Orange, CA, USA). Before beginning the procedure, we have to calculate the DNA volume that we will use, accordingly to its concentration and the quantity that we want to have (1000ng), and then we have to add sterile distilled water to the calculated DNA volume of each sample, until we reach the final volume of 20μL.

Briefly, the procedure comprises (*Figure 9*): bisulfite-mediated conversion of unmethylated cytosines, using temperature denaturation (thermocycler) to complement chemical denaturation; binding of the converted single-stranded DNA to the membrane of a spin column; washing; desulfonation of membrane-bound DNA; washing of the membrane-bound DNA to remove desulfonation agent; elution of the pure converted DNA from the spin column in 60μL

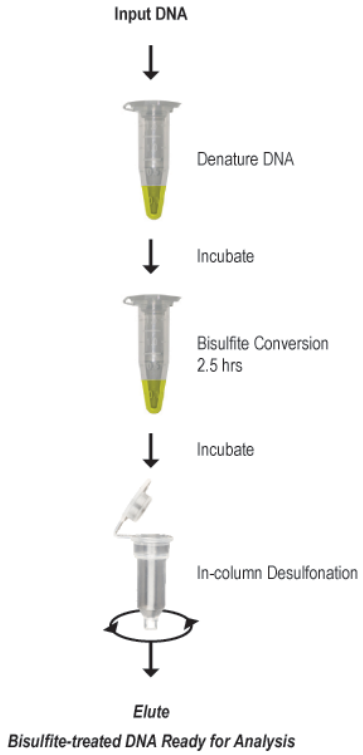

Figure 9 - The bisulfite conversion procedure using EZ DNA methylation Gold™ kit (Zymo Research). (Adapted from www.zymoresearch.com)

of sterile distilled water. Finally, the bisulfite modified DNA was stored at -80ºC, until further use.

2.3. Real-time fluorescence-based methylation-specific PCR

After sodium bisulfite conversion, DNA was used as template for Real-time fluorescence-based methylation-specific PCR (qMSP) using locus-specific PCR primers and probes, specified in the Appendix I.

The relative level of methylated DNA for each gene in each sample was determined using the following formula: [(target gene/ *ACTB* (beta-actin) x 1000] [128]. Fluorogenic QMSP assays were carried out using Platinum Taq (Invitrogen, Carlsbad, CA) for DNA extracted from paraffin-embedded tissues or TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems) for DNA extracted from urines in a reaction volume of 20µL, containing 900 nM of forward and reverse primers; 200 nM of probe; and 2 µL of bisulfite modified DNA as a template, in 96-well plates in an Applied Biosystems 7500 Sequence Detector (Perkin Elmer, Foster City, CA). *Figure 10* explains the assay principle: the 5' to 3' nuclease activity of *Taq* DNA polymerase cleaves the probe and releases the reporter, whose fluorescence can be detected by the laser detector of the Real-Time PCR System; after crossing a fluorescence detection threshold, the PCR amplification results in a fluorescence signal proportional to the amount of PCR product generated.

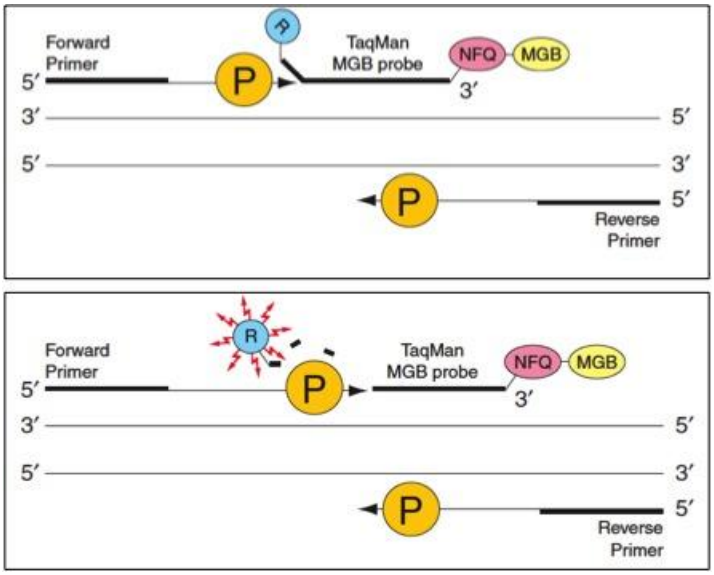

Figure 10. TaqMan technology chemistry: A – TaqMan MGB probe (with a reporter – R – at the 5' end and a quencher at the 3' end – NFQ) specific to the sequence anneals to DNA. B – During the amplification, DNA polymerase (P) is responsible for the cleavage of the probe, whose reporter emits fluorescence detected by Real-Time PCR System. (Adapted from TaqMan Gene Expression Assay Protocol provided by Applied Biosystems)

PCR was performed in separate wells for each primer/probe set and each sample was run in triplicate. Additionally, multiple water blanks were included as negative controls, and six serial dilutions (dilution factor of 5x) of a fully methylated bisulfite modified universal DNA control (in vitro methylated human DNA, Chemicon) were included in each plate to generate a standard curve.

A run was considered valid when the slope of each standard curve was above - 3.92, corresponding to PCR efficiency of greater than 80%; the R2 of at least 4 relevant data points exceeded 0,99; and no amplification in the negative controls. To ensure that there was no amplification in the negative controls, the water blanks and the standards of each plate were run in a 2% agarose gel electrophoresis.

3. Cell Culture and Treatment with Epigenetic Modulating Drugs

To assess the importance of methylation and, possibly, other epigenetic mechanisms in the biological development of UTUC, representative transitional carcinoma cell lines were selected and exposed to epigenetic modulating drugs. The methylation status of the used cell lines was previously determined in the prior study [88].

Two transitional carcinoma (J82 and TCCSUP) and one squamous carcinoma (SCaBER) cell lines were selected for subsequent studies (ATCC – American Type Culture Collection, MD, USA). Regarding their methylation status, SCaBER was used as a specificity control because it has lower methylation levels than transitional cell lines. SCaBER, J82 and TCCSUP cell lines were kindly provided by Dr. Sidransky, form Johns Hopkins University, USA.

All the cell lines were grown in the recommended medium – Eagle's Minimum Essential Medium (MEM) (GIBCO®, Invitrogen, Carlsbad, CA) – and supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO®, Invitrogen, Carlsbad, CA) and 1% Penicillin-Streptomycin (P-S) (GIBCO®, Invitrogen, Carlsbad, CA). Cell lines were subcultured using Trypsin (GIBCO®, Invitrogen, Carlsbad, CA) to harvest them as many times as necessary to obtain the desired number of 75 cm^3 cell culture flasks. Several tests for *Mycoplasma sp*. contamination were performed (PCR Mycoplasma Detection Set, Clontech Laboratories, Oxford, UK).

The cell lines were grown until 20 to 30% of confluence was reached in 75cm^3 cell culture flasks. Then, medium containing the corresponding drug(s) - a pharmacologic inhibitor of DNMTs, 5-aza-2-deoxycytidine (DAC) (Sigma-Aldrich®, Germany) and/or a pan-inhibitor of HDAC, Trichostatin A (TSA) (Sigma-Aldrich®, Germany) - was added accordingly. Culture medium and appropriate drug(s) were renewed every day according to the schedule represented in *Table 5*. The mock cells served as controls as they were submitted to the medium change procedure but were not exposed to any drug. All the treatments were done in triplicate.

On the fourth day of the treatment schedule, the cells were harvested by trypsinization and centrifuged. Then, either pellets were washed in PBS 1x and stored at -80°C for RNA extraction, or immediately processed for Protein extraction or ChIP analysis.

Table 5 - Schedule of cell lines treatment with the epigenetic modulating drugs DAC and TSA.

	0h	24h	48h	72h
Mock	15mL Medium	15mL Medium	15mL Medium	Harvest
1µM DAC	15mL Medium + 1.5µL DAC	15mL Medium + 1.5µL DAC	15mL Medium + 1.5µL DAC	Harvest
1µM DAC + 0.5 µM TSA	15mL Medium + 1.5µL DAC	15mL Medium + 1.5µL DAC	15mL Medium + 1.5µL DAC + 7.5 µL TSA	Harvest
0.5 µM TSA	15mL Medium	15mL Medium	15mL Medium + 7.5 µL TSA	Harvest

4. Molecular Processing of Drug Exposed Cells

4.1. RNA Extraction

Total RNA was extracted from cell lines pellets using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual.

Briefly, pellets were thawed on ice and 1mL of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) was added. Cells were homogenized using a syringe, and left for 5 min at room temperature to ensure the complete dissociation of nucleoprotein complexes. Then, all content of the tubes was transferred to 1.5mL RNAse-free tubes with 200µL of chloroform (Merck, Germany) previously added, vortexed and incubated for 3 min at room temperature, followed by a centrifugation for 15 min at 10,600rpm at 4ºC. At this point, the mixture consisted of a lower red, an interphase and a colorless aqueous phase, where RNA remained, so it was collected into a fresh 1.5mL RNAse-free tube placed on

ice. Next, 500µL of isopropyl alcohol were added to this content, and the tubes were placed at room temperature for 10 min to precipitate RNA. Following this, tubes were centrifuged for 10 min at 10,600rpm at 4ºC and the supernatant was carefully eliminated without disturbing the pellet. Finally, 1mL of 75% ethanol was added to wash RNA pellets using the vortex followed by one more centrifugation. Supernatant was eliminated and the RNA pellets were air-dried until alcohol evaporation.

RNA pellets were eluted, for at least 30 min, in a variable volume of RNA Storage Solution (1 mM Sodium Citrate, pH 6.4) (Ambion, Applied Biosystems, Foster City, CA, USA) according to pellet size. The eluted RNA was then evaluated for concentration and quality using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and stored at -80ºC, until the cDNA synthesis procedure.

4.2. cDNA Synthesis

In this step, and using the previous obtained RNA, cDNA was synthetized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Briefly, the following components were added to a RNAse-free PCR tube on ice: 1 µg of template RNA, 2 µL of 10x RT Buffer, 0.8 µL of 25x dNTP Mix (100 mM), 2 µL of 10x RT Random Primers, 1 µL of RNAse Inhibitor, 1 µL of MultiScribeTM Reverse Transcriptase, and the appropriate volume of DEPC-treated water (deionized, nuclease-free water) (MP Biomedicals LLC, Solon, OH, USA) to complete a total volume of 20 µL. The reverse transcription reaction was performed in Veriti® Thermal Cycler (Applied Biosystems) during the following incubation: 25ºC for 10 min, 37ºC for 120 min and 85ºC for 5 min. The newly synthesized cDNA samples were then diluted in 180 µL of DEPC-treated water.

All this procedure was also applied to Stratagene® QPCR Human Reference Total RNA (containing a mixture of RNA from ten different cell lines) (Stratagene, La Jolla, CA, USA) that was used as control for the Gene Expression Assays described further. In this particular case, cDNA was diluted in 100 µL of DEPC-treated water, and then stored at -20ºC (as the previous samples).

4.3. Protein Extraction

Protein extraction from whole-cell lysates was obtained using the Radio Immuno Precipitation Assay (RIPA) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

In brief, the growth medium was removed from the 75cm^3 cell culture flask by aspiration and the cells were washed with 1x phosphate-buffered saline (PBS) to remove residual medium. Then, 300 μL of RIPA buffer (10 μL of PMSF solution, 10 μL of sodium orthovanadate solution and 20 μL of protease inhibitor cocktail solution per mL of 1x RIPA Lysis Buffer) was added, and the flasks were quickly scrapped to promote cell lyses and removal. This cell lysate was then transferred into a 1.5 mL-tube and incubated for 30 min on ice, following a centrifugation for 30 min at 13,000 rpm at 4°C. The respective supernatant was then collected into a new 1.5 mL-tube.

Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific Inc., Bremen, Germany). The mechanism of action of this assay is based on a colorimetric detection and quantitation of total protein by bicinchoninic acid (BCA). Protein concentrations were determined with reference to standards of bovine serum albumin (BSA). The resulting purple-colored reaction product exhibits a strong absorbance at 562 nm, which is nearly linear with increasing protein concentrations over a broad working range. A series of dilutions of known concentration were prepared from BSA and assayed together with our samples.

In brief, 25 μL of each standard or sample and 200 μL of the working reagent (50:1, Reagent A:B) were added to a 1.5 mL-tube and subsequently incubated at 37°C for 30 min. The content of the tubes was then transferred into a 96-well plate (Ratiolab, Dreieich, Germany) and the absorbance was measured at 562 nm on a microplate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany). Proteins were then stored at -20°C until further use.

5. Quantitative Gene Expression Analysis

cDNA from all cell lines samples was used as the template for real-time PCR reaction to quantify the three genes transcripts. The VIM gene expression assays (Hs00185584_m1 from Applied Biosystems, Foster City, CA, USA) and the endogenous controls assay *GUSB* and *HPRT* (Hs99999908_m1 and Hs01003267_m1 from Applied Biosystems, Foster City, CA, USA) were used. These assays are based on the same technology previously described for qMSP assay.

The *GUSB* and *HPRT* assays were used to normalize cDNA input. All expression assays were performed separately in 96-well plates in a 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the recommended protocol. Briefly, in each well 9 μL of previously synthesized cDNA, 1 μL of Taqman® Gene Expression Assay and 10 μL of Taqman® Gene Expression Master Mix (Applied Biosystems) were added. The reactions were performed in 96-well plates in a 7500 Real-Time PCR System (Applied Biosystems) using the conditions indicated by the manufacturer: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 15 sec and 60°C for 1 min. All samples were run in triplicate and two negative controls (water blanks) were included in each plate. The standard curve method was used for quantitation. cDNA previously synthesized from Stratagene® QPCR Human Reference total RNA (Agilent Technologies) was used to prepare five consecutive cDNA dilutions (dilution factor of 10x) used as standards on each plate. Results were analyzed using the Sequence Detector Software version 1.2.3 (Applied Biosystems, Foster City, CA, USA).

The mean quantity of our gene of interest expression levels in each sample was normalized against mean quantity of *GUSB* and *HPRT* combined expression levels (Gene Expression Level= (Gene Mean Quantity / (*GUSB* & *HPRT*) Mean Quantity)).

6. Western-Blot

VIM protein expression was evaluated by Western Blot using a specific antibody.

In short, 30 μg of protein from each cell line were resuspended in loading buffer and denatured at 95°C for 5 min. Proteins were then separated by SDS-PAGE on 10% polyacrylamide gels at 120V at room temperature, and subsequently blotted onto Protran nitrocellulose transfer membranes (Whatman, Dassel, Germany) at 50V for 1h at 4°C. After electroblotting, the membranes were incubated in blocking buffer [3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST)] for 3h at room temperature with agitation. The membranes were then incubated overnight at 4°C with the primary monoclonal mouse antibody for VIM (Dako, Denmark) diluted 1:750 in blocking buffer. After three washing steps with TBST, the membranes were incubated for 1h at room temperature with a horseradish peroxidase conjugated secondary anti-mouse antibody (Bio-Rad, München, Germany) diluted 1:3000 in blocking buffer. After washing with TBST, the membranes were developed using Immun-Star WesternC Chemiluminescent Kit (Bio-Rad) and exposed to Amersham Hyperfilm (GE Healthcare).

To ensure equal loading of protein, the membranes were stripped and reprobed with an antibody against the loading control (ACTB). The membranes were incubated at room temperature for 15 min with 20 mL of EzWay Antibody Erasing Buffer (Koma Biotech, Seoul, Korea) under vigorous shaking, followed by at least 5 washes with bidistilled water (B. Braun).

After this step, membranes were incubated with a monoclonal mouse antibody against ACTB (Sigma-Aldrich) diluted 1:8000 in blocking buffer (5% nonfat dry milk in TBST) for 30 min at room temperature. After 3 x washing, the membranes were then incubated for 15 min at room temperature with a horseradish peroxidase conjugated secondary anti-mouse antibody (Bio-Rad) diluted 1:3000 in blocking buffer.

7. Chromatin Immunoprecipitation

A chromatin immunoprecipitation assay (ChIP) was performed using EZ-Magna ChIPTM G – One-Day Chromatin Immunoprecipitation Kit (Millipore, MA, USA) according to manufacturer's instructions, using the previously mentioned J82 cell line, after treatment with epigenetic modulating drugs. This ChIP procedure may be divided into 5 important steps, as we may see in *Figure 11*.

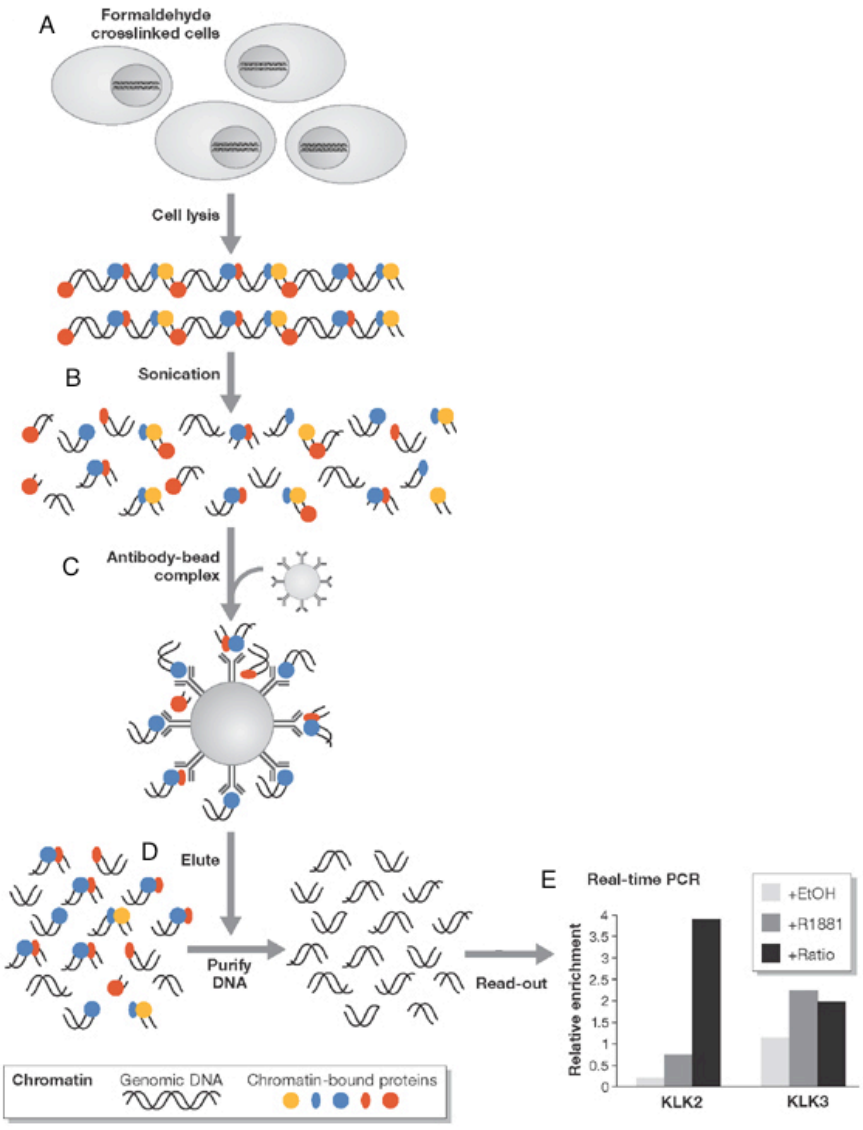

Figure 11. Five steps summary of chromatin immunoprecipitation (ChIP) methodology: (A) in vivo crosslinking and lysis, (B) sonication to shear DNA, (C) immunoprecipitation of crosslinked protein/DNA, (D) reverse crosslink of protein/DNA complexes to free DNA and its purification, and (E) example of an analysis of a gene ChIP enrichment by real-time quantitative PCR. (Adapted from [129])

On the first step – *in vivo* crosslinking and lysis - formaldehyde was added to each cell culture flask until a final concentration of 1% was reached, and incubated for 10 min at room temperature. Next, 2mL of 10x glycine were added in each flask in order to quench unreacted formaldehyde, and after a 5 min incubation at room temperature, flasks were placed on ice. Then, culture medium was carefully removed and cells were 2 x washed with 20mL of cold 1x PBS. Finally, cells were scrapped and resuspended into tubes containing 2mL of cold 1x PBS and 1x Protease Inhibitor Cocktail II (PIC), which were then centrifuged to pellet the cells, and remove supernatant. Cells were then putted on ice for 15min after being resuspended in 0.5mL of 1x Cell Lysis Buffer and 1x PIC, with vortex shaking every 5 min. Tubes were centrifuged again (800 x g for 5 min, at 4ºC), supernatant was removed and 0.5mL of 1x Nuclear Lysis Buffer and 1x PIC were added to resuspend the pellet.

At this time, sonication is needed do shear the DNA. This step was performed for 20 min, using 300μL of resuspended pellet, on iced water with a Bioruptor® Standard (Diagenode, Philadelphia, PA, USA), and with cycles of 20 sec with sonication ON, followed by 50 sec with sonication OFF. Sonicated chromatin was stored into 50μL aliquots at -80ºC, until further use.

After this, and in order to validate the sonication performance, 5μL of sonicated chromatin were incubated at 37ºC for 30 min, with 10μg of RNase (Sigma-Aldrich, Germany), following an addition of 1μL of Proteinase K and incubation at 62ºC for 2h. After that, the respective sample was loaded into a 2% agarose gel and runned for 1h at 140V, and was observed in an ultraviolet transilluminator [Pharmacia Biotech ImageMaster VDS (Pharmacia Biotech, Bay Area)].

The third step of ChIP procedure is the immunoprecipitation of crosslinked protein/DNA. Each 50μL aliquot of sonicated chromatin was diluted in 450μL 1x Dilution Buffer and 2.25μL 1x PIC, into a new tube. To prepare the input control, 5μL of this solution was reserved into a new tube, and reserved at 4ºC until elution of protein/DNA complexes and reverse crosslink of protein/DNA complexes to free DNA, as described below.

Afterwards, to each tube of the previously made solution were added, separately, 10μL of anti-H2A.Z (ab4174, Abcam, Cambridge, UK), 10μL of anti-AcH2A.Z (ab18262, Abcam, Cambridge, UK), 5μL of anti-H3 (ab1791, Abcam, Cambridge, UK), 5μL of anti-AcH3 (06-599, Millipore, MA, USA), 5μL of anti-

H3K4me3 (ab8580, Abcam, Cambridge, UK), 5μL of anti-H3K27me3 (07-499, Millipore, MA, USA), 5μL of anti-H3K9me3 (07-442, Millipore, MA, USA), 5μL of anti-H3K36me2 (ab9049, Abcam, Cambridge, UK), 5μL of anti-H4 (ab70701, Abcam, Cambridge, UK), 5μL of anti-H4K20me3 (ab9053-100, Abcam, Cambridge, UK), 1μL of the positive control anti-RNA Polymerase II and 1μL of the negative control Normal Mouse IgG, the last two provided with the kit. After, 20μL of fully suspended protein G magnetic beads were added to the tubes, which were then incubated overnight with rotation, at 4°C.

On the next day, protein G magnetic beads were pelleted using a magnetic separator (Magma Grip Rack) (Millipore, MA, USA) and the supernatant was removed. After that, beads were fully resuspended and washed for 5 min, with constant rotation, with four different buffers in the following order:

1st. Low Salt immune Complex Wash Buffer;

2nd. High Salt Immune Complex Wash Buffer;

3rd. LiCl Immune Complex Wash Buffer;

4th. TE Buffer.

To each tube were added 100μL of ChIP Elution Buffer and 1μL of Proteinase K, following incubation for 2h at 62°C, with permanent shaking. Hereinafter, a 95°C incubatation during 10min was performed, and tubes were allowed to cool until they reached room temperature. Beads were separated using the magnetic separator and the supernatant was reserved into fresh microfuge tubes.

For each immunoprecipitation, 500μL of Bind Reagent "A" were added to a Spin Filter within a Collection Tube. Tubes were then centrifuged at 12,600 x g for 30 seconds, and the eluate was discarded (both filter and collection tube were saved). Next, the same process was repeated, this time with Wash Reagent "B". Finally, the Spin Filter was put into a fresh collection tube, 50 μL of Elution Buffer "C" were added directly onto the center of the white Spin Filter Membrane and a new centrifugation was performed (12,600 x g for 30 seconds). The Spin Filter was discarded and the Collection Tube containing the purified DNA was stored at -20°C until further use.

At this point, DNA was ready for real-time quantitative PCR. This later was performed in order to analyze the specific prost-translational histone marks nearby *VIM* gene promoters. Therefore three pairs of primers were designed for *VIM* gene, being the primers "A" those closer to the TSS and primers "C" those

that were farthest from the TSS. Information about the primers used is compelled in *Table 6*. About this, it is important to mention that, until now, we couldn't be able to optimize the second pair of primers, the "B" pair.

Table 6 – ChIP qRT-PCR primers features: sequence, distance from TSS and annealing temperature.

* Lack optimization

Gene	Primer		Sequence	Distance from TSS	$T_{anneling}$ (°C)
VIM	A	Forward	5' tagtgagcaggagaaagcacag 3'	398bp	60
		Reverse	5' aaagacaggacatggaggatgt 3'		
	B	Forward	5' cactatgttggctcactgcaac 3'	785bp	n/a*
		Reverse	5' acttgaggtcaggagttcgaga 3'		
	C	Forward	5' ctgaactgatacagtggcaagtga 3'	1268bp	60
		Reverse	5' tcaggatatgcatgccaaag 3'		

The assays were performed separately in 96-well plates in 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol. Briefly, in each well 2μL of sonicated chromatin, 10μL of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.25μL (Primers "A") or 0.5μL (Primers "C") of primers solution (forward + reverse) (10mM) and 7.75μL or 7.5μL(respectively) of DEPC-treated water were added. PCR conditions were those previously defined by manufacturer: 50°C for 2min, 95°C for 10min, 45 cycles at 95°C for 15sec and 60°C for 1min, 95°C for 15sec, 60°C for 20sec and, finally, 95°C for 15 sec.

All samples were run in triplicate and multiple water blanks were added to each plate as negative controls.

The results were analyzed using the 7500 Software for 7500 and 7500 Fast Real Time PCR Systems V2.0.6 (Applied Biosystems, Foster City, CA, USA). A run was considered valid under conditions previously described. Data normalization was conducted using the Input Percent Method, meaning that signals obtained from ChIP samples were divided by signals obtained from the input sample, the last one representing the amount of chromatin used for immunoprecipitation. The enrichment over the core histone (H3, H4 or H2A.Z) was then calculated for each histone mark.

8. Statistical Analysis

The frequency, median and interquartile range of *GDF15*, *TMEFF2* and *VIM* promoter methylation levels of normal tissue/control samples and UTUC tissue/urine samples were determined.

To categorize samples as methylated or unmethylated, a cutoff value was chosen based on the highest methylation ratio value obtained for the respective normal/control samples, ensuring perfect specificity of the assay.

The nonparametric Mann-Whitney U-test assessed differences in quantitative methylation values. Relationships between methylation ratios and gender, grade, and pathological stage were evaluated using the Mann-Whitney and Kruskall-Wallis tests, as applicable. Spearman nonparametric correlation test was performed to assess the association between age and methylation levels.

Specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the three-gene panel as a UTUC biomarker were determined. A receiver operator characteristics (ROC) curve was constructed by plotting the true positive (sensitivity) against the false-positive (1-specificity) rate, and the area under de curve (AUC) was calculated.

Differences in proportions test was performed to compare the diagnostic performance of methylation analysis compared to urine cytology.

Disease-specific survival curves (Kaplan–Meier with log rank test) were computed for standard variables (tumor stage and grade) and for categorized methylation status. A Cox-regression model comprising all significant variables (multivariable model) was computed to assess the relative contribution of each variable to the follow-up status. For multivariable testing, pathological stage and age were recoded into two groups (Ta–T1 versus T2–4; lower than 75 years versus higher than 75 years).

In cell lines, differences in VIM transcript as well as in histone marks levels among the treatments performed were determined using a One-Way Analysis of Variance (one-Way ANOVA) test, followed by a multiple comparison Dunnett's test, comparing all groups against the Mock.

Two-tailed P-values were derived from statistical tests, using a computer-assisted program (SPSS Version 20.0, Chicago, IL), and results were considered statistically significant at $P < 0.05$, with Bonferroni´s correction for multiple tests, when applicable.

RESULTS

1. Methylation analysis of UTUC tissues and performance of the methylation panel in urines

1.1. Tissue samples analysis

The majority of UTUC tissue samples were methylated at *GDF15*, *TMEFF2* and *VIM* promoters, and relative methylation levels were significantly higher when compared to normal urothelium (P = 0.022; P < 0.001; P < 0.001, respectively) (*Figure 12* and *Table 7*).

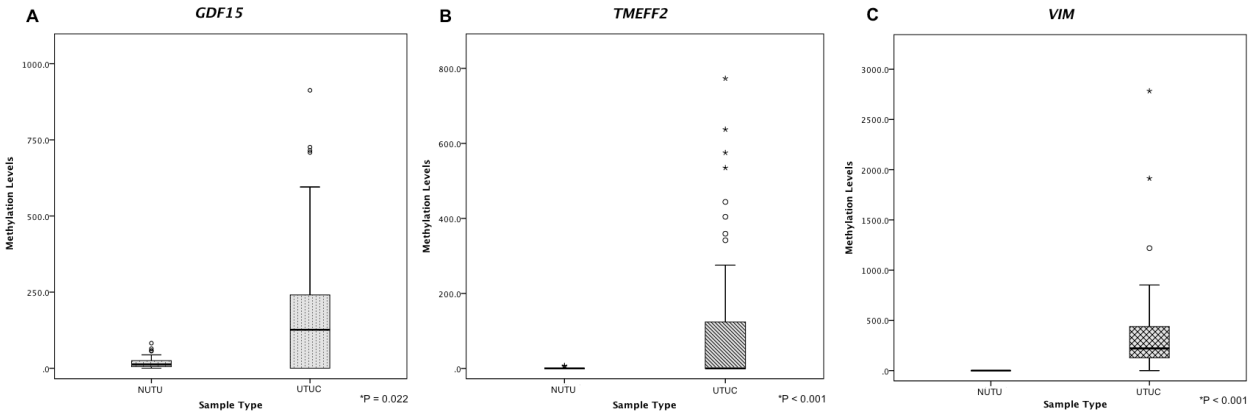

Figure 12. Box-plots of (A) *GDF15*, (B) *TMEFF2* and (C) *VIM* promoter methylation levels across both normal upper tract urothelium (NUTU) and upper tract urothelial carcinoma (UTUC) tissues (*Mann-Whitney U Test).

Table 7 - Frequency and distribution of promoter methylation levels in normal upper tract urothelium (NUTU) and upper tract urothelial carcinoma (UTUC) tissue samples measured by qMSP.

Gene	NUTU, n	NUTU med (IQR)	UTUC, n	UTUC med (IQR)	*P* value*
GDF15	0/36	24.89 (10.95 - 48.85)	32/57	252.74 (0.0 - 481.78)	**0.022**
TMEFF2	0/36	0.0 (0.0 - 0.0)	26/57	0.0 (0.0 - 123.49)	**<0.001**
VIM	0/36	0.0 (0.0 - 0.0)	54/57	220.25 (126.69 - 438.67)	**<0.001**

1.2. Urine samples analysis

The three-gene panel was then tested in a set of urine sediments from 22 UTUC patients and a mix of 20 subjects not carrying UTUC (controls). Methylation levels in UTUC urines were significantly higher than those of controls, for all genes (*Table 8*).

Table 8 - Frequency and distribution of promoter methylation levels in normal upper tract urothelium (NUTU) and upper tract urothelial carcinoma (UTUC) tissue samples measured by qMSP.

Gene	Control, n	Control med (IQR)	UTUC, n	UTUC med (IQR)	*P* value*
GDF15	0/20	0.0 (0.0 - 0.0)	17/22	3.79 (0.09 - 21.67)	**<0.001**
TMEFF2	0/20	0.0 (0.0 - 0.0)	15/22	6.33 (0.0 - 57.22)	**0.001**
VIM	0/20	0.0 (0.0 - 0.0)	18/22	31.28 (11.07 - 145.81)	**<0.001**

1.3. Biomarker Performance

The combined assessment of promoter-methylation of *GDF15, TMEFF2* and *VIM* allowed for discrimination of UTUC tissue samples from normal mucosa with 100% sensitivity and specificity, providing full diagnostic coverage (*Table 9*). These results were confirmed by ROC curve analysis, with an AUC of 1.000 [95% confidence interval (CI): 0.998 - 1.000, P < 0.001] (*Figure 13B*). Notably, the gene panel identified UTUC in urine with 91% sensitivity and 100% specificity. ROC curve analysis depicted an AUC of 0.923 [95% CI: 0.819 - 1.000, P < 0.001] (*Figure 13C*).

Table 9 - Performance of epigenetic biomarkers for the detection of upper tract urothelial carcinoma in tissue and urine sediments.

	Sensitivity % (n positive / n total)	Specificity % (n negative / n total)	PPV %	NPV %	Accuracy %
Tissue samples					
VIM	95 (54/57)	100 (36/36)	100	92	97
VIM / GDF15	98 (56/57)	100 (36/36)	100	97	99
VIM / GDF15 / TMEFF2	100 (57/57)	100 (36/36)	100	100	100
Urine samples					
VIM	82 (18/22)	100 (20/20)	100	83	91
VIM / GDF15	91 (20/22)	100 (20/20)	100	91	95
VIM / GDF15 / TMEFF2	91 (20/22)	100 (20/20)	100	91	95

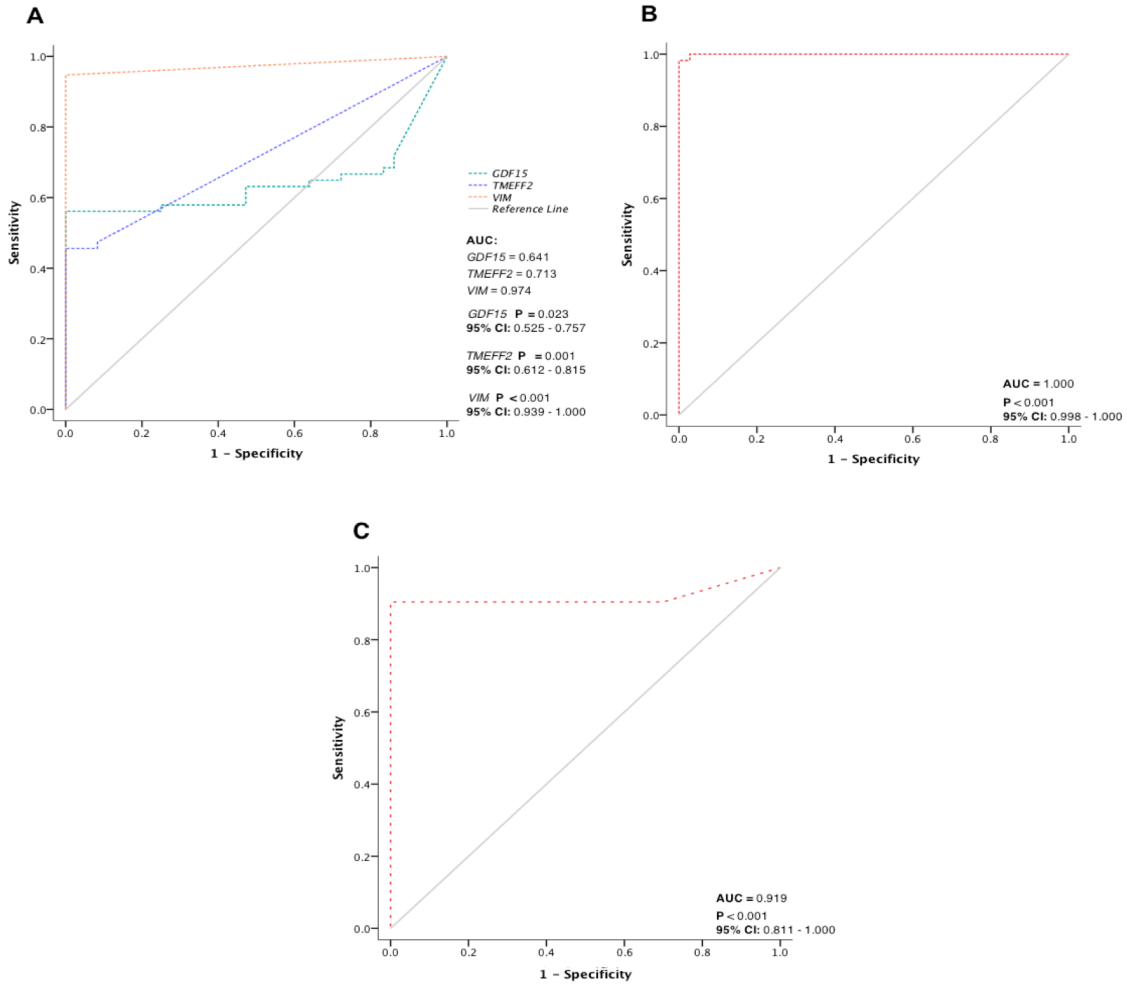

Figure 13. Receiver operating characteristic curve in upper tract urothelial carcinoma (UTUC) for each individual gene (*GDF15*, *TMEFF2*, and *VIM*) in tissues (A), and the combination of three genes (*GDF15*, *TMEFF2*, and *VIM*) in tissues (B), and in urines (C).

To ascertain the clinical usefulness of the methylation assay, its performance was compared with cytological diagnosis performed by an experienced cytopathologist, in 19 cases. Remarkably, the proportion of true positive cases using the panel of methylated genes was significantly higher than that of cytology ($P < 0.001$; *Figure 14*). Of 19 cases analyzed, only five were cytologically diagnosed as malignant, six as negative for malignancy, and eight were rendered as "inconclusive/suspicious for malignancy", corresponding to an overall sensitivity of only 26% (*Table 10*). Strikingly, the gene panel correctly identified 17 cases as malignant (89% sensitivity), whereas the remaining two cases were also cytologically diagnosed as negative.

Figure 14. Pie charts representing the diagnostic test sensitivity for the methylated gene panel - *GDF15*, *TMEFF2*, and *VIM* (A) and cytology (B), performed in urine samples from the same patients (n = 19) diagnosed with upper tract urothelial carcinoma. (*Differences in Proportions Test).

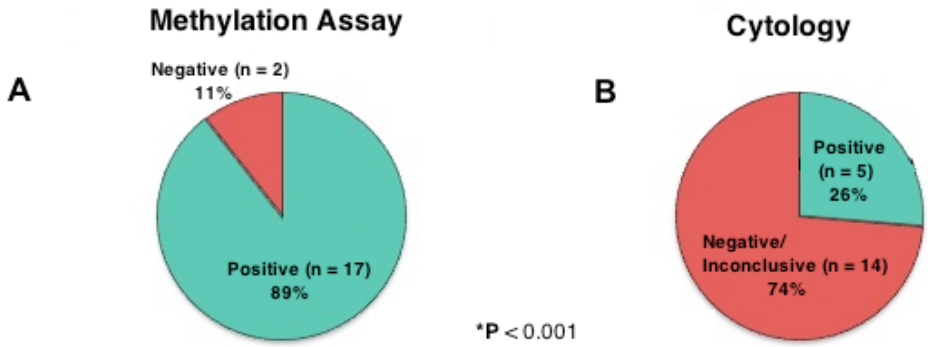

Table 10 - Comparison between results of methylated gene panel and cytology tests across urine samples from the same patient. A green, yellow or red box, indicate a positive, inconclusive or negative result, respectively.

	Methylation Assay	Cytology
#1		
#2		
#3		
#4		
#5		
#6		
#7		
#8		
#9		
#10		
#11		
#12		
#13		
#14		
#15		
#16		
#17		
#18		
#19		

2. Association between quantitative promoter methylation and clinicopathological parameters and survival analyses

No association was disclosed between *GDF15, TMEFF2* and *VIM* promoter methylation and patient's gender or pathological stage. A significant association was found for *GDF15* methylation levels and age in UTUC patients (R = 0.303, P = 0.022), but not in controls. Additionally, a significant association of *VIM* methylation levels and higher tumor grade (P = 0.015) was found, and *VIM* methylation levels differed between papillary high-grade and invasive UTUC (P = 0.006).

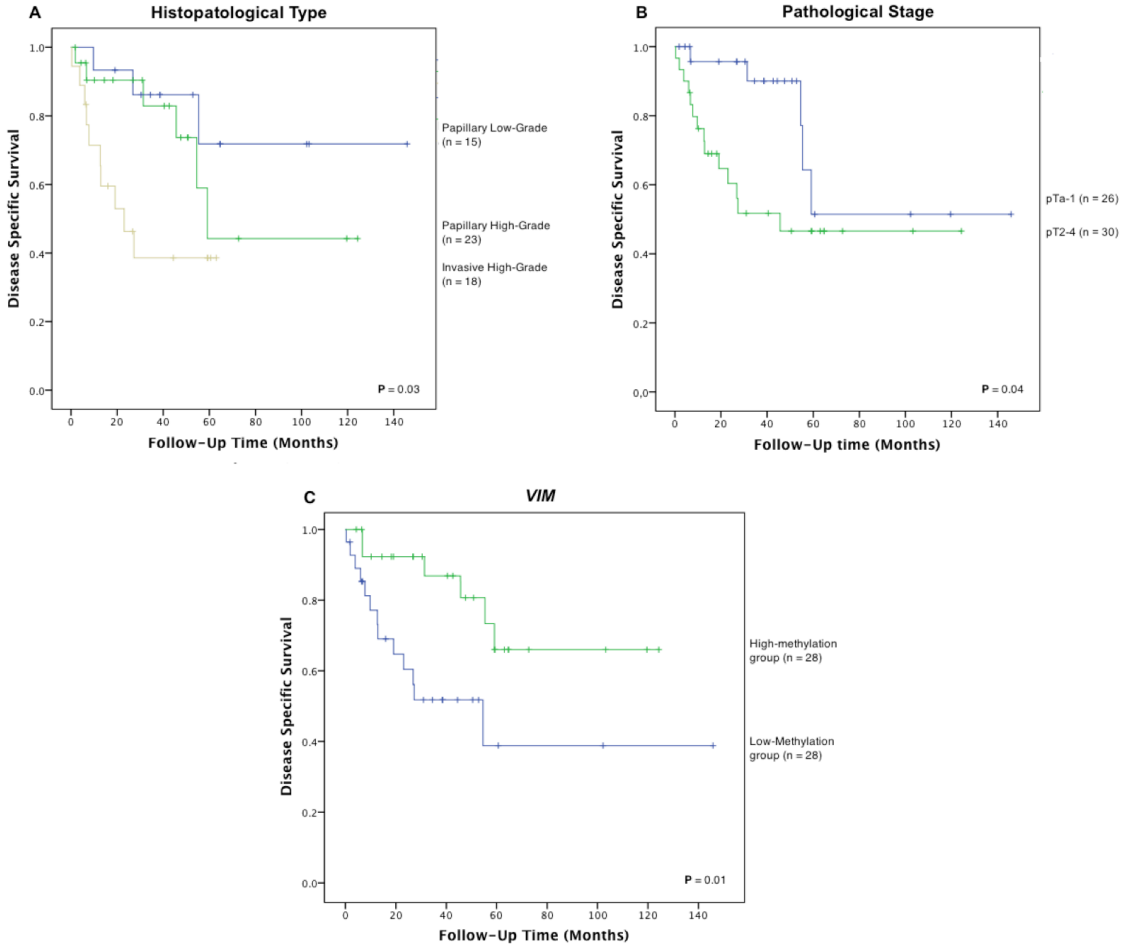

Figure 15. Disease-specific survival according to clinicopathological parameters [histopathological classification (A) and pathological stage (B)] and *VIM* promoter methylation status (C).

Of the 57 UTUC patients one was lost for follow-up. The median follow-up was 31 months (range: 1–146 months). At the time of the last follow-up, 23 patients were alive with no evidence of cancer, 9 patients were alive with cancer

progression, and 24 patients had deceased, 19 of which due to UTUC. For the purpose of survival analyses, all cases were coded based on gene methylation levels using the median value as cutoff. Disease-specific survival analysis showed that patients with low *VIM* promoter methylation levels had a significantly shorter overall survival (P = 0.013; *Figure 15C*), and a 3.3-fold increased hazard ratio (P = 0.019; *Table 11*). The same was not observed for *GDF15* and *TMEFF2* promoter methylation, thus we only further analyzed *VIM* methylation.

A poor outcome was depicted for patients with higher grade and pathological stage (*Figure 15A and 15B*; P = 0.03 and P = 0.04, respectively). In univariate Cox-regression analysis, both histopathological type and pathological stage, but not age or gender, carried prognostic information (P = 0.025 and P = 0.049; *Table 11*). When clinical and epigenetic variables were introduced in a Cox-regression model for disease-specific survival, pathological stage and *VIM* methylation status were selected in the final model as independent predictors of outcome. After patients were categorized according to pathological stage, low *VIM* methylation levels carried a 18-fold increased risk of cancer-related death (95% CI: 3.24–99.25, P < 0.001) in the advanced disease group (pT2-4). Disease-free survival analysis did not disclose any significant association.

Table 11 - Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of disease-specific survival for 56 upper tract urothelial carcinoma (UTUC) patients (PLG, papillary low-grade; PHG, papillary high-grade; IHG, invasive high-grade)

Disease-specific Survival	Variables	Hazard Ratio (HR)	95% CI* for OR	P
Univariate	**pTNM**	2.81	(1.01 - 7.83)	**0.049**
	Grade			
	PLG vs. PHG	1.79	(0.45 - 7.17)	0.411
	PLG vs. IHG	4.41	(1.21 - 16.11)	**0.025**
	Age	1.93	(0.69 - 5.40)	0.208
	Gender	0.36	(0.11 - 1.23)	0.104
	***VIM* methylation ≤ median**	3.26	(1.22 - 8.73)	**0.019**
Multivariate	**pTNM**	3.78	(1.18 - 12.07)	**0.025**
	Grade PLG vs. PHG	2.51	(0.60 - 10.56)	0.209
	PLG vs. IHG	3.02	(0.79 - 11.53)	0.106
	***VIM* methylation ≤ median**	5.97	(1.81 - 19.64)	**0.003**

3. Influence of the action of Epigenetic Modulating Drugs in VIM transcript and protein levels

3.1. Association between VIM Transcript and Promoter Methylation Levels, after the Action of Epigenetic Modulating Drugs

Given the previous results in patient's samples, we decided to investigate VIM specific role in the carcinogenesis of UTUC, using *in vitro* models for that purpose.

Previously, we have demonstrated that cell lines' exposure to DAC and/or TSA reduced methylation levels (data not shown) [88], however no assessment of *VIM* transcript levels has been yet performed. Thus, two transitional carcinoma cell lines (J82, TCCSUP) and one squamous cell carcinoma cell line (SCaBER) were exposed to these drugs, either alone or in combination, and expression levels were determined.

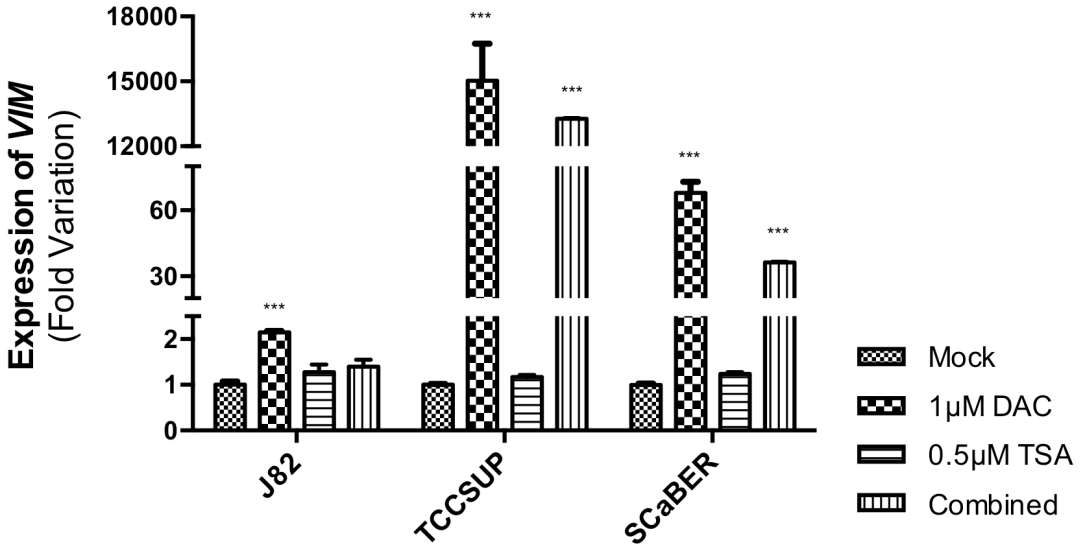

Figure 16. Transcript levels of VIM in J82, TCCSUP and SCaBER cell lines after exposure to the epigenetic modulating drugs DAC and/or TSA. The results are presented in fold variation in comparison to the experimental control. (Dunnett Test, ***p< 0.001)

As depicted in *Figure 16*, 1μM DAC exposure significantly increased *VIM* transcript levels in all the three cell lines ($p < 0.001$). The combined treatment with DAC and TSA also significantly increased *VIM* expression levels in TCCSUP and SCaBER cell lines ($p < 0.001$). Moreover, cell lines treated with TSA alone did not show any variation of VIM transcript levels, comparing with untreated cells.

3.2. Influence of Epigenetic Modulating Drugs in VIM Protein Levels

To confirm the impact of DAC on *VIM* re-expression in J82, TCCSUP and SCaBER cell lines, the respective protein levels were also assessed by Western Blot. Similarly to transcript levels, VIM protein levels significantly increased in all cell lines after DAC exposure, alone and in combination with TSA (*Figure 17*).

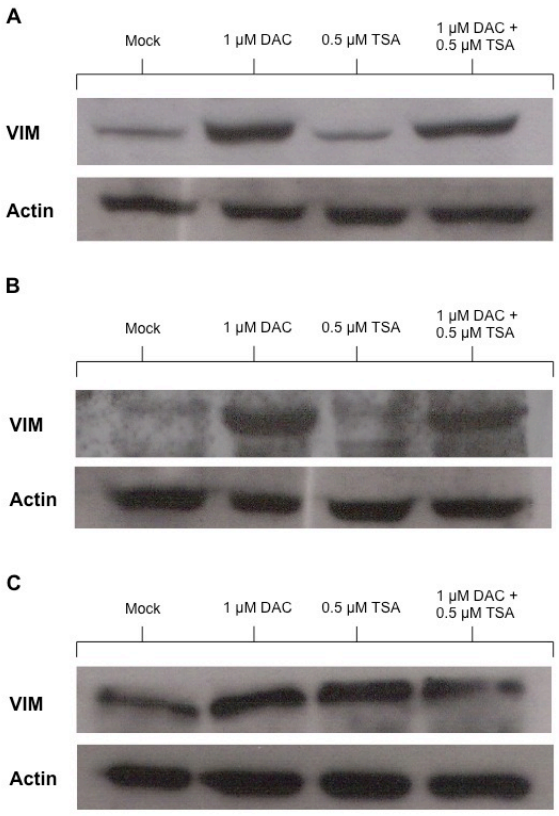

Figure 17. VIM protein expression analysis by Western Blot in (A) J82, (B) TCCSUP and (C) SCaBER cell lines exposed to epigenetic modulating drugs DAC and TSA. The picture is representative of three independent experiments.

Surprisingly, VIM protein levels in SCaBER cells (*Figure 17C*) treated with TSA (0.5μM concentration) seem to have increased significantly, following the opposite trend of the respective transcript levels.

4. Chromatin Immunoprecipitation Analysis

After treatment with epigenetic modulating drugs DAC and TSA, ChIP was performed in J82 cell line. At this point, it is only worth continuing using the transitional carcinoma cell lines, because it is our biological model of interest, and SCaBER cells are no longer needed as a methylation/expression control. TCCSUP ChIP results were not obtained at this time.

Chromatin of treated cell lines was immunoprecipitated for several transcriptional activating histone marks (acH2A.Z, acH3, H3K36me2 and H3K4me3) and repression histone marks (H3K27me3, H3K9me3 and H4K20me3). Immunoprecipitation for H2A.Z, H3 and H4 was also performed. After ChIP, qRT-PCR was carried out to evaluate accumulation of marks along the VIM promoter region.

Primarily, we observed that DAC exposure increased H3, H4 and H2A.Z deposition across *VIM* promoter, and more specifically, the combined treatment seemed to be crucial in the enrichment of the above-mentioned histones at nucleosomes distant from the TSS (*Figure 18 A, G and I*). Furthermore, H4 and H2A.Z were practically absent in J82 untreated cells, whereas increased levels of these histones were found after cells exposure to epigenetic modulating drugs, especially upon exposure to combined drugs.

Interestingly, both H3K27me3, H3K9me3 and acH2A.Z levels were barely detectable on mock and drug treated cells, suggesting that this marks do not exist across *VIM* promoter (*Figure 18 D, E and J*).

Concerning H3 activation marks, acH3, H3K4me3 and H3K36me2, an increase was observed in nucleosomes distant to the TSS after the combined DAC and TSA exposure (*Figure 18 B, C and F*).

Contrarily, the deposition of the repressive mark H4K20me3 dramatically decreased after drugs exposure, across the entire *VIM* promoter (*Figure 18H*).

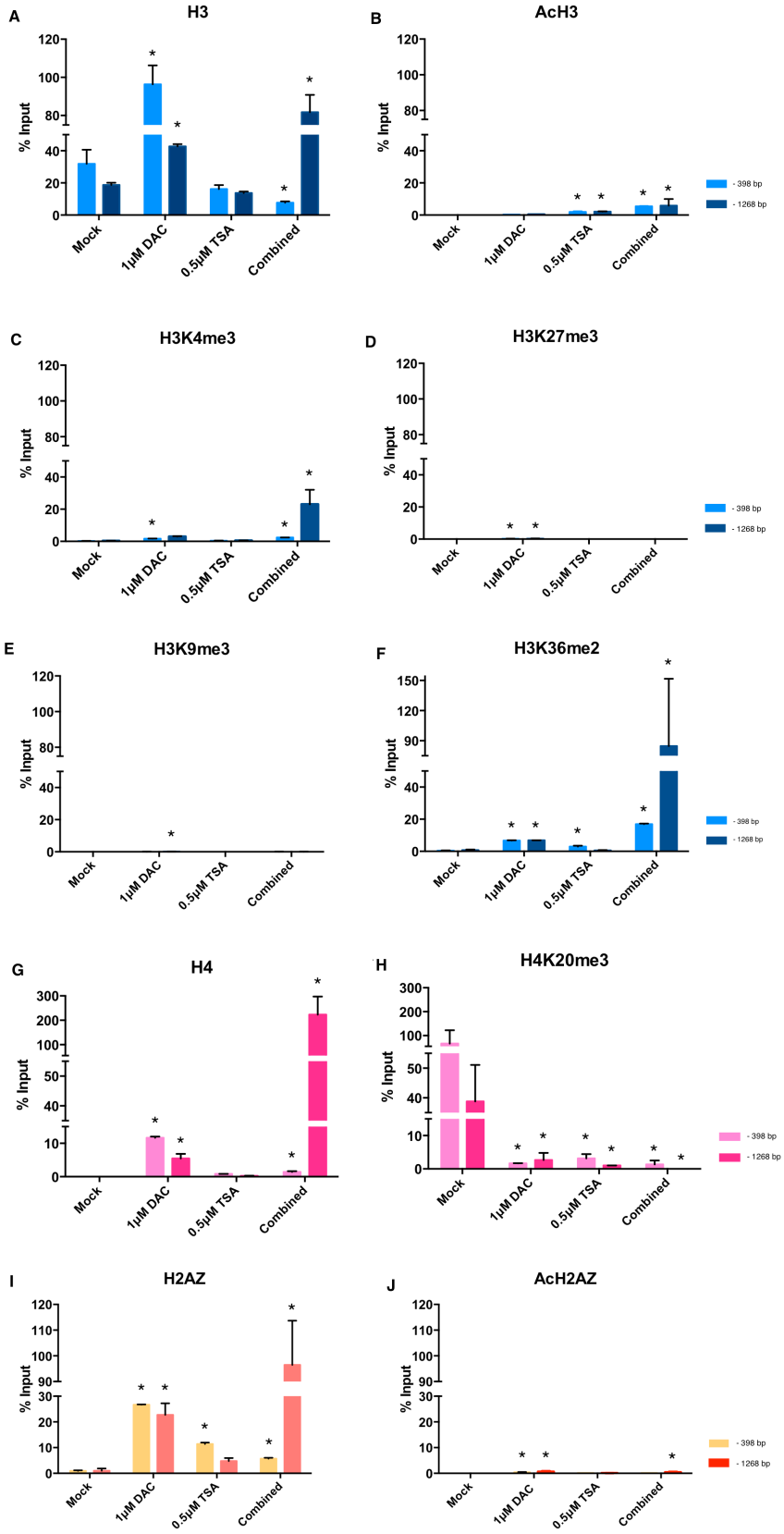

Figure 18. ChIP results for J82 cell line concerning (A) H3, (B) acH3, (C) H3K4me3, (D) H3K27me3, (E) H3K9me3, (F) H3K36me2, (G) H4, (H) H4K20me3, (I) H2A.Z and (J) acH2A.Z histones and histones marks across VIM promoter. Results are normalized with the input of total sonicated chromatin. (Dunnett's Test, *p < 0.002)

DISCUSSION

UTUCs are clinically aggressive neoplasms (60% are invasive at diagnosis, compared to 15-20% for bladder UCC), mostly due to late diagnosis, as UTUC is usually silent at its earliest stages and difficult to detect using ultrasonography and urine cytology. Thus, the development of novel, cost-effective and non-invasive methods for accurate diagnosis of UTUC are likely to have a significant impact in patient management. Gene promoter methylation, a cancer hallmark, has shown promise for accurate detection of urological cancers (especially those of the prostate and bladder) and may thus be of clinical usefulness [130]. We have recently identified a panel of markers (*GDF15, TMEFF2* and *VIM* methylation), which detects bladder UCC with high sensitivity and specificity, both in tissue and urine [88]. Owing to the morphological and genomic similarities with UTUC [125], we hypothesized that the same gene panel might show a comparable performance.

Remarkably, this panel identified UTUC with 100% and 91% sensitivity in tissue and urine samples, respectively, maintaining perfect specificity in both. These results constitute a significant step forward in the detection of UTUC and provide a basis for the development of an assay to be used in clinical practice. In fact, owing to the difficult accessibility of the UUT, current methods used to detect a neoplasm in this anatomical location are mostly ineffective. Indeed, our study demonstrates that urine cytology may hardly be considered a diagnostic modality since it only allowed for the diagnosis of 26% of cases. Although higher sensitivity has been reported elsewhere, reaching 40% [131], cases diagnosed by cytology are usually muscle-invasive, thus carrying an unfavorable prognosis [131, 132].

On the other hand, high specificity is also very important because most of individuals to which this test might be offered are not carriers of UTUC, and thus, the requirement for other procedures to confirm an abnormal result of the test, most of which would be invasive, has to be minimized. Finally, it should be emphasized that this gene panel is able to discriminate UCC from renal cell carcinoma, confirming another previous observations made by our group [133], thus providing valuable information for cases in which ultrasonography is unable to discriminate tumors of renal pelvic from renal parenchymal origin. The

purpose of including renal cell tumors, as well as prostate cancer, as controls in urine samples was to demonstrate the tumor-specificity of the gene panel. Indeed, UTUC, renal cell tumors and prostate carcinoma are amenable to detection in urine samples and occur in similar age spectra. Thus, it would be important for this gene methylation test to accurately predict urothelial carcinoma, discriminating from other common tumors of the genitourinary tract.

Interestingly, survival analysis showed that low *VIM* promoter methylation levels independently predict poor disease-specific survival in UTUC patients, particularly those with high-stage disease. Thus, the gene panel is able to convey not only diagnostic, but also, prognostically important information. Because detection of UTUC based on *GDF15, TMEFF2* and *VIM* relies on the identification of higher promoter methylation levels in UCC compared to normal urothelium, the association of low *VIM* promoter methylation levels with worse prognosis might seem paradoxical. However, it should be recalled that *VIM* encodes for vimentin, an intermediate filament characteristic of cells with mesenchymal phenotype, not expressed in most normal epithelia (including urothelium) neither in most carcinomas [134]. However, *VIM* de-novo expression or overexpression has been reported in various epithelial cancers, including those of prostate [135], breast [136] and lung [137], associating with increased tumor growth, invasion and poor prognosis. In these instances, vimentin expression has been associated with epithelial to mesenchymal transition (EMT), a biological process that is associated with an invasive phenotype [134]. Thus, we speculate that during early UUT carcinogenesis, the *VIM* promoter is progressively methylated and the gene is kept silenced, as in normal urothelium, whereas in a subset of UTUC, methylation is decreased, allowing for aberrant vimentin expression, due to stimuli leading to EMT. As a consequence, these tumors would be more prone to local invasion and systemic dissemination, thus fostering disease progression and reducing disease-specific survival.

One of the major limitations of our study consists on the relatively small number of cases analyzed. However, it should be noted that UTUC is a relatively uncommon neoplasm and we report on the casuistic of a single-center. This also justifies the fact that in only 19 cases we had information on the cytological diagnosis, which corresponds to the cases diagnosed more recently during the collection period of this study. Thus, a larger and, ideally, multicenter study,

should be performed to validate our findings. Moreover, this gene panel is unable to discriminate upper from lower urinary tract UCC. Thus, only after exclusion of bladder and urethral UCC should a diagnosis of UTUC be considered following a positive test. However, owing to the similarities between UTUC and lower urinary tract UCC, the identification of specific markers is difficult. Some studies proposed that microsatellite instability [123], *CDH1* [138], and *FXYD3* gene expression [139] might provide such discrimination but none has been replicated by other research teams, yet. Thus, positive testing would require additional work-up of the urinary tract.

Additionally, we investigated whether this gene is epigenetically regulated in urothelial carcinomas.

As expected, *VIM* transcription levels increased significantly after exposure to DAC, a DNMTs inhibitor, i.e. a demethylation agent, in both tested transitional carcinoma cell lines. These results compare well with those of methylation obtained previously for the same cell lines [88]. Moreover, protein levels of VIM also followed the same trend as observed for transcript levels for same treatment conditions. Importantly, the increased transcript levels were corroborated by ChIP assay results, which demonstrated an increase in histone activating marks, such as acH3, H3K4me3 and H3K36me2 far from the TSS, after DAC and TSA exposure, with concomitant decrease of the repressive histone mark H4K20me3 [140]. Moreover, regarding acH2AZ low deposition across the TSS, our results are in accordance with previous studies on PCa cell lines, in which genes highly methylated displayed lower levels of this mark, whereas a gain of acH2AZ mark was associated with gene activation in cells lines treated with epigenetic modulating drugs [141]. Therefore, our results suggest that epigenetic modulating drugs are able to induce VIM re-expression.

Bringing together our data, we can postulate that epigenetic mechanisms are responsible of *VIM* deregulation in urothelial carcinomas. However, it is important to replicate these same results in other transitional carcinoma cell lines to assure its validity.

CONCLUSIONS AND FUTURE PERSPECTIVES

The molecular characterization and the management of UCs and UTUCs have progressed considerably in the past few years. The identification of the different mechanisms of carcinogenesis in UCs of the bladder and of the UUT has incited many researchers to focus on this issue. Moreover, the development of novel diagnostic and prognostic biomarkers for UTUC, which might allow for its identification, is required to provide a non-invasive, specific and sensitive method, easy to perform, and cost-effective.

In this thesis we were able to demonstrate that *GDF15, TMEFF2* and *VIM* promoter methylation accurately discriminates UTUC from normal urothelium, prostate and renal cell carcinoma, both in tissue and urine samples. Furthermore, low *VIM* methylation levels predicted poor prognosis, especially in high-stage disease. Thus, this gene panel may assist in the clinical management of UTUC patients.

Importantly, we also showed that VIM expression is epigenetically regulated in urothelial carcinoma.

To ascertain the utility of *VIM* expression in clinical samples and to clarify how *VIM* epigenetic deregulation affects urothelial cells' neoplastic transformation, we need to perform additional tasks, specifically:

- ⇒ Perform immunohistochemical analysis in the series of UTUC tissues;
- ⇒ Induction of *VIM* expression on methylated transitional carcinoma cell lines using a full-length human VIM cDNA clone;
- ⇒ Perform functional assays on the transfected cells: cell viability, apoptosis and invasion assays;
- ⇒ Accomplish *in vivo* studies of tumors' formation.

BIBLIOGRAPHY

1. Waddington CH. (1939) An Introduction to Modern Genetics. London: George Alien & Unwin Ltd.

2. Feinberg AP, Tycko B. (2004) The history of cancer epigenetics. Nat Rev Cancer;4(2):143-53.

3. Holliday R. Epigenetics: a historical overview. (2006) Epigenetics. 1(2):76-80.

4. Feinberg AP. (2010) Genome-scale approaches to the epigenetics of common human disease. Virchows Arch. 456(1):13-21.

5. Sharma S, Kelly TK, Jones PA. (2010) Epigenetics in cancer. Carcinogenesis. 31(1):27-36.

6. Tost J. (2009) DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. Methods Mol Biol. 507:3-20.

7. Sawan C, Vaissiere T, Murr R, Herceg Z. (2008) Epigenetic drivers and genetic passengers on the road to cancer. Mutat Res. 642(1-2):1-13.

8. Yan MS, Matouk CC, Marsden PA. (2010) Epigenetics of the vascular endothelium. J Appl Physiol. 109: 916–926.

9. Mulero-Navarro S, Esteller M. (2008) Epigenetic biomarkers for human cancer: the time is now. Crit Rev Oncol Hematol. 68(1):1-11.

10. Ehrlich M, Gama-Sosa MA, Huang LH, et al. (1982) Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. Nucleic Acids Res. 10(8):2709-21.

11. Herman JG, Baylin SB. (2003) Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 349(21):2042-54.

12. Bernstein BE, Meissner A, Lander ES. (2007) The mammalian epigenome. Cell. 128(4):669-81.

13. Esteller M, Herman JG. (2002) Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J Pathol. 196(1):1-7.

14. Gronbaek K, Hother C, Jones PA. (2007) Epigenetic changes in cancer. APMIS. 115(10):1039-59.

15. Jones PA, Baylin SB. (2007) The epigenomics of cancer. Cell. 128(4):683-92.

16. Takai D, Jones PA. (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A. 99(6):3740-5.

17. Antequera F, Bird A. (1993) CpG islands. EXS. 64:169-85.

18. Vaissiere T, Sawan C, Herceg Z. (2008) Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res. 659(1-2):40-8.

19. Clark SJ, Melki J. (2002) DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene. 21(35):5380-7.

20. Klose RJ, Bird AP. (2006) Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci. 31(2):89-97.

21. Cheng X, Blumenthal RM. (2008) Mammalian DNA methyltransferases: a structural perspective. Structure. 16(3):341-50.

22. Turek-Plewa J, Jagodzinski PP. (2005) The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell Mol Biol Lett. 10(4):631-47.

23. Sasai N, Defossez PA. (2009) Many paths to one goal? The proteins that recognize methylated DNA in eukaryotes. Int J Dev Biol. 53(2-3):323-34.

24. Bird A. (2002) DNA methylation patterns and epigenetic memory. Genes Dev. 16(1):6-21.

25. Gartler SM, Riggs AD. (1983) Mammalian X-chromosome inactivation. Annu Rev Genet. 17:155-90.

26. Scarano MI, Strazzullo M, Matarazzo MR, D'Esposito M. (2005) DNA methylation 40 years later: Its role in human health and disease. J Cell Physiol. 204(1):21-35.

27. Fraga MF, Esteller M. (2007) Epigenetics and aging: the targets and the marks. Trends Genet. 23(8):413-8.

28. Miranda TB, Jones PA. (2007) DNA methylation: the nuts and bolts of repression. J Cell Physiol. 213(2):384-90.

29. Esteller M. (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet. 8(4):286-98.

30. Kornberg RD, Lorch Y. (1999) Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell. 98(3):285-94.

31. Portela A, Esteller M. (2010) Epigenetic modifications and human disease. Nat Biotechnol. 28(10):1057-68.

32. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature. 389(6648):251-60.

33. Kouzarides T. (2007) Chromatin modifications and their function. Cell. 128(4):693-705.

34. Huertas D, Sendra R, Munoz P. (2009) Chromatin dynamics coupled to DNA repair. Epigenetics. 4(1):31-42.

35. Luco RF, Pan Q, Tominaga K, Blencowe BJ, Pereira-Smith OM, Misteli T. (2010) Regulation of alternative splicing by histone modifications. Science. 327(5968):996-1000.

36. Jenuwein T, Allis CD. (2001) Translating the histone code. Science. 293(5532):1074-80.

37. Lennartsson A, Ekwall K. (2009) Histone modification patterns and epigenetic codes. Biochim Biophys Acta. 1790(9):863-8.

38. Strahl BD, Allis CD. (2000) The language of covalent histone modifications. Nature. 403(6765):41-5.

39. Grunstein M. (1997) Histone acetylation in chromatin structure and transcription. Nature. 389(6649):349-52.

40. Bhaumik SR, Smith E, Shilatifard A. (2007) Covalent modifications of histones during development and disease pathogenesis. Nat Struct Mol Biol. 14(11):1008-16.

41. Santos-Rosa H, Caldas C. (2005) Chromatin modifier enzymes, the histone code and cancer. Eur J Cancer. 41(16):2381-402.

42. Li B, Carey M, Workman JL. (2007) The role of chromatin during transcription. Cell. 128(4):707-19.

43. Rice JC, Allis CD. (2001) Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol. 13(3):263-73.

44. Mills AA. (2010) Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat Rev Cancer. 10(10):669-82.

45. Lopez J, Percharde M, Coley HM, Webb A, Crook T. (2009) The context and potential of epigenetics in oncology. Br J Cancer. 100(4):571-7.

46. Bartel DP. MicroRNAs: target recognition and regulatory functions. (2009) Cell. 136(2):215-33.

47. Filipowicz W, Bhattacharyya SN, Sonenberg N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 9(2):102-14.

48. Ambros V. (2004) The functions of animal microRNAs. Nature. 431(7006):350-5.

49. Bartel DP. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 116(2):281-97.

50. Valeri N, Vannini I, Fanini F, Calore F, Adair B, Fabbri M. (2009) Epigenetics, miRNAs, and human cancer: a new chapter in human gene regulation. Mamm Genome. 20(9-10):573-80.

51. Hanahan D, Weinberg RA. (2000) The hallmarks of cancer. Cell. 100(1):57-70.

52. Nowell PC. (1976) The clonal evolution of tumor cell populations. Science. 194(4260):23-8.

53. Campbell LL, Polyak K. (2007) Breast tumor heterogeneity: cancer stem cells or clonal evolution? Cell Cycle. 6(19):2332-8.

54. Jones PA, Baylin SB. (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet. 3(6):415-28.

55. Baylin SB, Jones PA. (2011) A decade of exploring the cancer epigenome - biological and translational implications. Nat Rev Cancer. 11(10):726-34.

56. Hirst M, Marra MA. (2009) Epigenetics and human disease. Int J Biochem Cell Biol. 41(1):136-46.

57. Sakatani T, Kaneda A, Iacobuzio-Donahue CA, et al. (2005) Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. Science. 307(5717):1976-8.

58. Eden A, Gaudet F, Waghmare A, Jaenisch R. (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science. 300(5618):455.

59. Gaudet F, Hodgson JG, Eden A, et al. (2003) Induction of tumors in mice by genomic hypomethylation. Science. 300(5618):489-92.

60. Bestor TH. (2005) Transposons reanimated in mice. Cell. 122(3):322-5.

61. Walsh CP, Chaillet JR, Bestor TH. (1998) Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat Genet. 20(2):116-7.

62. Nishigaki M, Aoyagi K, Danjoh I, et al. (2005) Discovery of aberrant expression of R-RAS by cancer-linked DNA hypomethylation in gastric cancer using microarrays. Cancer Res. 65(6):2115-24.

63. Knudson AG, Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A. 68(4):820-3.

64. Esteller M. (2005) Aberrant DNA methylation as a cancer-inducing mechanism. Annu Rev Pharmacol Toxicol. 45:629-56.

65. Esteller M. (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum Mol Genet. 16 Spec No 1:R50-9.

66. Cedar H, Bergman Y. (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet. 10(5):295-304.

67. Fullgrabe J, Kavanagh E, Joseph B. (2011) Histone onco-modifications. Oncogene. 30(31):3391-403.

68. Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE, Robson CN. (2004) Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate. 59(2):177-89.

69. Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M. (2004) Stem cells and cancer; the polycomb connection. Cell. 118(4):409-18.

70. Khare SP, Sharma A, Deodhar KK, Gupta S. (2011) Overexpression of histone variant H2A.1 and cellular transformation are related in N-nitrosodiethylamine-induced sequential hepatocarcinogenesis. Exp Biol Med (Maywood). 236(1):30-5.

71. Calin GA, Dumitru CD, Shimizu M, et al. (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 99(24):15524-9.

72. Chivukula RR, Mendell JT. (2008) Circular reasoning: microRNAs and cell-cycle control. Trends Biochem Sci. 33(10):474-81.

73. Deng S, Calin GA, Croce CM, Coukos G, Zhang L. (2008) Mechanisms of microRNA deregulation in human cancer. Cell Cycle. 7(17):2643-6.

74. Ruan K, Fang X, Ouyang G. (2009) MicroRNAs: novel regulators in the hallmarks of human cancer. Cancer Lett. 285(2):116-26.

75. Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC. (2006) Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res. 66(3):1277-81.

76. Mack GS. (2006) Epigenetic cancer therapy makes headway. J Natl Cancer Inst. 98(20):1443-4.

77. Minucci S, Pelicci PG. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer. 6(1):38-51.

78. Yoo CB, Jones PA. (2006) Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov. 5(1):37-50.

79. Thompson CA. (2006) Vorinostat approved for rare lymphoma. Am J Health Syst Pharm. 63(22):2168.

80. Campas-Moya C. (2009) Romidepsin for the treatment of cutaneous T-cell lymphoma. Drugs Today (Barc). 45(11):787-95.

81. Johnstone RW. (2002) Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov. 1(4):287-99.

82. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet. 21(1):103-7.

83. Zhu WG, Otterson GA. (2003) The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. Curr Med Chem Anticancer Agents. 3(3):187-99.

84. Henrique R, [VL, Jeronimo C. (2007) Methylation-based biomarkers for early detection of urological cancer. Crit Rev Oncog. 13(4):265-82.

85. Shi H, Wang MX, Caldwell CW. (2007) CpG islands: their potential as biomarkers for cancer. Expert Rev Mol Diagn. 7(5):519-31.

86. Laird PW. (2003) The power and the promise of DNA methylation markers. Nat Rev Cancer. 3(4):253-66.

87. Laird PW. (2005) Cancer epigenetics. Hum Mol Genet. 14 Spec No 1:R65-76.

88. Costa VL, Henrique R, Danielsen SA, et al. (2010) Three epigenetic biomarkers, GDF15, TMEFF2, and VIM, accurately predict bladder cancer from DNA-based analyses of urine samples. Clin Cancer Res. 16(23):5842-51.

89. Hsiao EC, Koniaris LG, Zimmers-Koniaris T, Sebald SM, Huynh TV, Lee SJ. (2000) Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. Mol Cell Biol. 20(10):3742-51.

90. Chen WD, Han ZJ, Skoletsky J, et al. (2005) Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst. 97(15):1124-32.

91. Zou H, Harrington JJ, Shire AM, et al. (2007) Highly methylated genes in colorectal neoplasia: implications for screening. Cancer Epidemiol Biomarkers Prev. 16(12):2686-96.

92. Shirahata A, Sakata M, Sakuraba K, et al. (2009) Vimentin methylation as a marker for advanced colorectal carcinoma. Anticancer Res. 29(1):279-81.

93. Tsunoda S, Smith E, De Young NJ, et al. (2009) Methylation of CLDN6, FBN2, RBP1, RBP4, TFPI2, and TMEFF2 in esophageal squamous cell carcinoma. Oncol Rep. 21(4):1067-73.

94. Jin Z, Cheng Y, Gu W, et al. (2009) A multicenter, double-blinded validation study of methylation biomarkers for progression prediction in Barrett's esophagus. Cancer Res. 69(10):4112-5.

95. Herzlinger D. (2011) Upper urinary tract pacemaker cells join the GLI club. J Clin Invest. 121(3):836-8.

96. Wu XR. (2009) Biology of urothelial tumorigenesis: insights from genetically engineered mice. Cancer Metastasis Rev. 28(3-4):281-90.

97. Munoz JJ, Ellison LM. (2000) Upper tract urothelial neoplasms: incidence and survival during the last 2 decades. J Urol. 164(5):1523-5.

98. Ploeg M, Aben KK, Kiemeney LA. (2009) The present and future burden of urinary bladder cancer in the world. World J Urol. 27(3):289-93.

99. Roupret M, Babjuk M, Comperat E, et al. (2013) European Guidelines on Upper Tract Urothelial Carcinomas: 2013 Update. Eur Urol.

100. Babjuk M, Burger M, Zigeuner R, et al. (2013) EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2013. Eur Urol. 2013;64(4):639-53.

101. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62(1):10-29.

102. Shariat SF, Favaretto RL, Gupta A, et al. (2011) Gender differences in radical nephroureterectomy for upper tract urothelial carcinoma. World J Urol. 29(4):481-6.

103. Eble JL, Sauter G, Epstein JI, Sesterhenn IA. (2004) Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs: WHO Classification of Tumours: World Health Organization.

104. Colin P, Koenig P, Ouzzane A, et al. (2009) Environmental factors involved in carcinogenesis of urothelial cell carcinomas of the upper urinary tract. BJU Int. 104(10):1436-40.

105. Pommer W, Bronder E, Klimpel A, Helmert U, Greiser E, Molzahn M. (1999) Urothelial cancer at different tumour sites: role of smoking and habitual intake of analgesics and laxatives. Results of the Berlin Urothelial Cancer Study. Nephrol Dial Transplant. 14(12):2892-7.

106. Shinka T, Miyai M, Sawada Y, Inagaki T, Okawa T. (1995) Factors affecting the occurrence of urothelial tumors in dye workers exposed to aromatic amines. Int J Urol. 2(4):243-8.

107. Debelle FD, Vanherweghem JL, Nortier JL. (2008) Aristolochic acid nephropathy: a worldwide problem. Kidney Int. 74(2):158-69.

108. Grollman AP, Shibutani S, Moriya M, et al. (2007) Aristolochic acid and the etiology of endemic (Balkan) nephropathy. Proc Natl Acad Sci U S A. 104(29):12129-34.

109. Margulis V, Youssef RF, Karakiewicz PI, et al. (2010) Preoperative multivariable prognostic model for prediction of nonorgan confined urothelial carcinoma of the upper urinary tract. J Urol. 184(2):453-8.

110. Mian C, Mazzoleni G, Vikoler S, et al. (2010) Fluorescence in situ hybridisation in the diagnosis of upper urinary tract tumours. Eur Urol. 58(2):288-92.

111. Clements T, Messer JC, Terrell JD, et al. (2012) High-grade ureteroscopic biopsy is associated with advanced pathology of upper-tract urothelial carcinoma tumors at definitive surgical resection. J Endourol. 26(4):398-402.

112. Ouzzane A, Colin P, Xylinas E, et al. (2011) Ureteral and multifocal tumours have worse prognosis than renal pelvic tumours in urothelial carcinoma of the upper urinary tract treated by nephroureterectomy. Eur Urol. 60(6):1258-65.

113. Lughezzani G, Burger M, Margulis V, et al. (2012) Prognostic factors in upper urinary tract urothelial carcinomas: a comprehensive review of the current literature. Eur Urol. 62(1):100-14.

114. Shariat SF, Godoy G, Lotan Y, et al. (2010) Advanced patient age is associated with inferior cancer-specific survival after radical nephroureterectomy. BJU Int. 105(12):1672-7.

115. Muramaki M, Miyake H, Terakawa T, Kusuda Y, Fujisawa M. (2011) Expression profile of E-cadherin and N-cadherin in urothelial carcinoma of the upper urinary tract is associated with disease recurrence in patients undergoing nephroureterectomy. Urology. 78(6):1443 e7-12.

116. Nakanishi K, Hiroi S, Tominaga S, et al. (2005) Expression of hypoxia-inducible factor-1alpha protein predicts survival in patients with transitional cell carcinoma of the upper urinary tract. Clin Cancer Res. 11(7):2583-90.

117. Kamai T, Takagi K, Asami H, Ito Y, Arai K, Yoshida KI. (2000) Prognostic significance of p27Kip1 and Ki-67 expression in carcinoma of the renal pelvis and ureter. BJU Int. 86(1):14-9.

118. Leibl S, Zigeuner R, Hutterer G, Chromecki T, Rehak P, Langner C. (2008) EGFR expression in urothelial carcinoma of the upper urinary tract is associated with disease progression and metaplastic morphology. APMIS. 116(1):27-32.

119. Vershasselt-Crinquette M, Colin P, Ouzzane A, et al. (2012) Assessment of human epidermal growth factor receptor 2 status in urothelial carcinoma of the upper urinary tract: a study using dual-color in situ hybridization and immunohistochemistry. Appl Immunohistochem Mol Morphol. 20(4):363-6.

120. Nakanishi K, Kawai T, Hiroi S, et al. (1999) Expression of telomerase mRNA component (hTR) in transitional cell carcinoma of the upper urinary tract. Cancer. 86(10):2109-16.

121. Lynch HT, de la Chapelle A. (2003) Hereditary colorectal cancer. N Engl J Med. 348(10):919-32.

122. Roupret M, Catto J, Coulet F, et al. (2004) Microsatellite instability as indicator of MSH2 gene mutation in patients with upper urinary tract transitional cell carcinoma. J Med Genet. 41(7):e91.

123. Roupret M, Fromont G, Azzouzi AR, et al. (2005) Microsatellite instability as predictor of survival in patients with invasive upper urinary tract transitional cell carcinoma. Urology. 65(6):1233-7.

124. Catto JW, Azzouzi AR, Rehman I, et al. (2005) Promoter hypermethylation is associated with tumor location, stage, and subsequent progression in transitional cell carcinoma. J Clin Oncol. 23(13):2903-10.

125. Zhang Z, Furge KA, Yang XJ, Teh BT, Hansel DE. (2010) Comparative gene expression profiling analysis of urothelial carcinoma of the renal pelvis and bladder. BMC Med Genomics. 3:58.

126. Edge SBB, D.R.; Compton, C.C.; Fritz, A.G.; Greene, F.L.; Trotti, A. American Joint Committee on Cancer cancer staging manual. ed. 7th Philadelphia: Lippincott-Raven Publishers; 2010.

127. Pearson H, Stirling D. (2003) DNA extraction from tissue. Methods Mol Biol. 226:33-4.

128. Jeronimo C, Henrique R, Hoque MO, et al. (2004) Quantitative RARbeta2 hypermethylation: a promising prostate cancer marker. Clin Cancer Res. 10(12 Pt 1):4010-4.

129. Massie CE, Mills IG. (2008) ChIPping away at gene regulation. EMBO Rep. 9(4):337-43.

130. Phe V, Cussenot O, Roupret M. (2009) Interest of methylated genes as biomarkers in urothelial cell carcinomas of the urinary tract. BJU Int. 104(7):896-901.

131. Messer J, Shariat SF, Brien JC, et al. (2011) Urinary cytology has a poor performance for predicting invasive or high-grade upper-tract urothelial carcinoma. BJU Int. 108(5):701-5.

132. Brien JC, Shariat SF, Herman MP, et al. (2010) Preoperative hydronephrosis, ureteroscopic biopsy grade and urinary cytology can improve prediction of advanced upper tract urothelial carcinoma. J Urol. 184(1):69-73.

133. Costa VL, Henrique R, Ribeiro FR, et al. (2007) Quantitative promoter methylation analysis of multiple cancer-related genes in renal cell tumors. BMC Cancer. 7:133.

134. Satelli A, Li S. (2011) Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci. 68(18):3033-46.

135. Singh S, Sadacharan S, Su S, Belldegrun A, Persad S, Singh G. (2003) Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer. Cancer Res. 63(9):2306-11.

136. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M. (2007) Vimentin and epithelial-mesenchymal transition in human breast cancer--observations in vitro and in vivo. Cells Tissues Organs. 185(1-3):191-203.

137. Al-Saad S, Al-Shibli K, Donnem T, Persson M, Bremnes RM, Busund LT. (2008) The prognostic impact of NF-kappaB p105, vimentin, E-cadherin and Par6 expression in epithelial and stromal compartment in non-small-cell lung cancer. Br J Cancer. 99(9):1476-83.

138. Eltz S, Comperat E, Cussenot O, Roupret M. (2008) Molecular and histological markers in urothelial carcinomas of the upper urinary tract. BJU Int. 102(5):532-5.

139. Zhang Z, Pang ST, Kasper KA, et al. (2011) FXYD3: A Promising Biomarker for Urothelial Carcinoma. Biomark Insights. 6:17-26.

140. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. (2007) Genome regulation by polycomb and trithorax proteins. Cell. 128(4):735-45.

141. Valdes-Mora F, Song JZ, Statham AL, et al. (2012) Acetylation of H2A.Z is a key epigenetic modification associated with gene deregulation and epigenetic remodeling in cancer. Genome Res. 22(2):307-21.

APPENDIX I

Sequences of the primers and probes used in the quantitative methylation-specific PCR experiments, with sizes and amplicons generated.

Primer Set		Sequence	Product size, bp	$T_{Annealing}$ ºC
ACTB_QMSP	Forward Primer	5' TGG TGA TGG AGG AGG TTT AGT AAG T 3'	133	60
	Reverse Primer	5' AAC CAA TAA AAC CTA CTC CTC CCT TAA 3'		
	Probe	FAM 5' ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA 3' TAMRA		
GDF15_QMSP	Forward Primer	5' TCG GCG GTT ATT TGT ATT TGC 3'	101	60
	Reverse Primer	5' CGT CGA AAA CAA CCG AAA CA 3'		
	Probe	5' FAM - TTT TCG AGG TTT TTC G - MGB 3'		
TMEFF2_QMSP	Forward Primer	5' GTT CGG GGT TAC GCG C 3'	83	60
	Reverse Primer	5' TTC GCC TCA CTC TCC GCT 3'		
	Probe	5' FAM - TCG GAT TTC GTT TTC GGT AG - MGB 3'		
VIM_QMSP	Forward Primer	5' TTC GGG AGT TAG TTC GCG TT 3'	108	60
	Reverse Primer	5' ACC GCC GAA CAT CCT ACG A 3'		
	Probe	5' FAM - TCG TCG TTT AGG TTA TCG T - MGB 3'		

APPENDIX II

ORIGINAL PUBLICATION

Sara Monteiro-Reis, Luís Leça, Mafalda Almeida, Luís Antunes, Paula Monteiro, Paula C. Dias, António Morais, Jorge Oliveira, Rui Henrique, Carmen Jerónimo.

"Accurate detection of upper tract urothelial carcinoma in tissue and urine by means of quantitative GDF15, TMEFF2 and VIM promoter methylation"

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Accurate detection of upper tract urothelial carcinoma in tissue and urine by means of quantitative *GDF15*, *TMEFF2* and *VIM* promoter methylation

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KEYWORDS
Detection
Methylation
Upper tract urothelial cancer
Urine markers

Abstract ***Aim of the study:*** Upper tract urothelial carcinoma (UTUC) accounts for 5–10% of all urothelial tumours. It is mostly diagnosed at advanced stages, entailing a worse prognosis, owing to the lack of early and specific symptoms as well as of effective diagnostic tools.
We previously identified a panel of epigenetic biomarkers (*GDF15*, *TMEFF2* and *VIM* promoter methylation) that accurately identifies bladder cancer in urine. Herein, we assessed the performance of the same panel for UTUC detection and prognosis, in tissue and urine.
Material and methods: Methylation levels of reference and target genes were determined using real-time quantitative methylation-specific polymerase chain reaction (MSP) in bisulphite-modified DNA of 57 UTUC tissues, 36 normal upper tract urothelium (NUTUs), 22 urines from UTUC suspects and 20 urines from controls.
Receiver operator characteristics (ROC)-curve analysis was performed to determine the performance of the biomarker panel and survival analyses were conducted to evaluate their prognostic value.
Results: Methylation levels of *GDF15*, *TMEFF2* and *VIM* were significantly higher in UTUC

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compared to NUTUs ($P = 0.022$; $P < 0.001$; $P < 0.001$, respectively). The panel accurately identified UTUC with 100% and 91% sensitivity, corresponding to an area under the curve of 1.000 and 0.923 in tissue and urines, respectively, with 100% specificity. Low *VIM* promoter methylation levels independently predicted poor disease-specific survival.

Conclusions: *GDF15*, *TMEFF2* and *VIM* promoter methylation allows for accurate identification of UTUC, in tissue and urine and *VIM* methylation provides relevant prognostic information, especially in high-stage disease. This assay may improve the clinical management of UTUC patients.

1. Introduction

Urothelial (transitional) cell carcinomas (UCC), arising from the specialised epithelium lining upper (renal pelvis and urethers) and lower (bladder and urethra) urinary tract [1], are the fourth most common tumours after prostate (or breast), lung and colorectal cancers [2,3]. Upper tract UCCs (UTUC) are less common (5–10% of all cases), mostly affecting adult males in their seventies [4] and, contrarily to bladder-UCC, most (60%) are invasive at diagnosis, mainly because they are clinically silent until an advanced stage [5]. Moreover, current diagnostic tools for UTUC display low sensitivity and/or are invasive, precluding their use for early detection [6].

Epigenetic changes are common events in cancer, and DNA methylation, usually associated with gene expression loss, is the better characterised [7]. Early occurrence of DNA methylation in cancer coupled with its chemical and biological stability renders it a potential diagnostic tool. Indeed, DNA methylation-based biomarkers have shown promise for clinical application in Oncology, from early detection/diagnosis to the assessment of prognosis and prediction of therapeutic response [8]. Importantly, DNA methylation assays can be performed on small biopsy archived frozen or paraffin-embedded samples obtained during routine diagnostic work-up of patients, as well as on the soluble genomic DNA found in peripheral blood and other fluids from cancer patients, offering the possibility of non-invasive molecular screening for many malignancies, including urological tumours [9].

In a previous work, a panel of three epigenetic biomarkers – promoter methylation of *GDF15*, *TMEFF2* and *VIM* – allowed for accurate identification of bladder UCC, with 100% sensitivity and specificity in tissue samples and 94% sensitivity and 100% specificity in urine samples [10]. Because bladder UCC and UTUC display clinical and genomic similarities [11], we hypothesised that the same panel of biomarkers might be useful for non-invasive, early detection of UTUC. Thus, we firstly evaluated the performance of quantitative *GDF15*, *TMEFF2* and *VIM* promoter methylation in a consecutive series of tissue samples from UTUC and subsequently determined the feasibility of detecting UTUC in voided urine samples using the same assay.

2. Materials and methods

2.1. Patients and tumour sample collection

Fifty-seven UTUC samples were obtained from a consecutive series of patients diagnosed and treated with radical nephroureterectomy or ureterectomy at the Portuguese Oncology Institute – Porto, Portugal, between 2000 and 2011, with no previous history of bladder cancer. Tissues were routinely fixed and paraffin-embedded for standard pathologic examination, allowing for tumour classification and World Health Organisation (WHO)/International Society of Urological Pathology (ISUP) grading [1] and staging [12]. Additionally, an independent set of 36 paraffin-embedded normal upper tract urothelium from renal cell carcinoma patients was used as controls. Relevant clinical data were collected from clinical charts (Table 1). This study was approved by the institutional review board (IRB-CES-IPOFG-EPE 019/08).

2.2. Urine sample collection and processing

Voided urine samples (one per patient) were collected from 22 patients with UTUC who were diagnosed and treated between 2006 and 2012 at the Portuguese Oncology Institute – Porto, Portugal. A set of 20 voided urine samples from patients with renal cell carcinoma ($n = 10$), prostate carcinoma ($n = 7$) and healthy blood donors with no personal or family history of cancer ($n = 3$), were also collected and used for control purposes (Table 2). Informed consent was obtained for urine sample collection of patients and controls (IRB-CES-IPOFG-EPE 019/08). Urine storage and processing conditions were standardised: each sample was immediately centrifuged at 4000 rpm for 10 min, the pelleted sediment was then washed twice with phosphate-buffered saline and stored at –80 °C.

2.3. Nucleic acids isolation, bisulphite treatment and quantitative methylation-specific polymerase chain reaction (qMSP)

From each case, a representative paraffin block was selected and the tumour area was delimited. Then, 10-20 serial 7-micrometres thick sections were cut and placed on glass slides, and macrodissected. Subsequently,

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Table 1
Clinical and histopathological parameters of patients with upper tract urothelial tumours and normal upper tract urothelium.

Clinicopathological features	UTUC	NUTU
Patients, *n*	57	36
Gender, *n* (%)		
Male	39 (68)	23 (64)
Female	18 (32)	13 (36)
Median age, years (range)	72 (52–85)	62 (49–82)
Pathological stage, *n* (%)		
pTa	4 (7)	n.a.
pT1	22 (38)	n.a.
pT2	13 (23)	n.a.
pT3	17 (30)	n.a.
pT4	1 (2)	n.a.
Grade, *n* (%)		
Papillary, low-grade	15 (26)	n.a.
Papillary, high-grade	23 (41)	n.a.
Invasive, high-grade	19 (33)	n.a.

Abbreviations: UTUC, upper tract urothelial carcinoma; NUTU, normal upper tract urothelium; n.a., not applicable.

Table 2
Clinical and histopathological parameters of patients with upper urinary tract urothelial tumours and gender and age distribution of the control set individuals [non-healthy donors (HD; $n = 3$), and renal cell tumour (RCT; $n = 10$) and prostate cancer (PCa; $n = 7$) patients], which provided urine samples for this study.

Clinicopathological features	UTUC	Control Set
Patients, *n*	22	20
Gender, *n* (%)		
Male	12 (54)	11 (55)
Female	10 (46)	9 (45)
Median age, years (range)	73 (53–86)	61 (48–83)
Grade, *n* (%)		
Papillary, low-grade	3 (14)	n.a.
Papillary, high-grade	12 (54)	n.a.
Invasive, high-grade	7 (32)	n.a.

Abbreviations: UTUC, upper tract urothelial carcinoma urine samples; n.a, not applicable.

samples were deparaffinised and digested with proteinase K (20 mg/ml, 50 μL). DNA from tissue and urine samples was extracted using standard phenol–chloroform [13], and concentrations were determined using ND-1000 Nanodrop (NanoDrop Technologies, Wilmington, United States of America [USA]). DNA samples were bisulphite modified using EZ DNA methylation – Gold kit (Zymo Research, Orange, CA), eluted in 60 μL of distiled water and stored at –80 °C. Bisulphite-modified DNA was used as template for qMSP using previously published locus-specific Polymerase Chain Reaction (PCR) primers and probes [10]. The relative level of methylated DNA for each gene in each sample was determined using the following formula: [(target gene/*ACTB* (beta-actin) × 1000] [14]. Fluorogenic QMSP assays were carried out using Platinum Taq (Invitrogen, Carlsbad, CA) for DNA extracted from paraffin-embedded tissues) or TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems) for DNA extracted from urines in a reaction volume of 20 μL, containing 900 nM of forward and reverse primers; 200 nM of probe; and 2 μL of bisulphite modified DNA as a template, in 96-well plates in an Applied Biosystems 7500 Sequence Detector (Perkin Elmer, Foster City, CA). PCR was performed in separate wells for each primer/probe set and each sample was run in triplicate. Additionally, multiple water blanks were included as negative controls, and six serial dilutions (dilution factor of 5×) of a fully methylated bisulphite modified universal DNA control (*in vitro* methylated human DNA, Chemicon) were included in each plate to generate a standard curve. A run was considered valid when previously reported criteria were met [10].

2.4. Statistical analysis

The frequency, median and interquartile range of *GDF15*, *TMEFF2* and *VIM* promoter methylation levels of normal tissue/control samples and UTUC tissue/urine samples were determined. To categorise samples as methylated or unmethylated, a cutoff value was chosen based on the highest methylation ratio value obtained for the respective normal/control samples, ensuring perfect specificity of the assay. The non-parametric Mann–Whitney *U*-test assessed differences in quantitative methylation values. Relationships between methylation ratios and gender, grade and pathological stage were evaluated using the Mann–Whitney and Kruskall–Wallis tests, as applicable. Spearman non-parametric correlation test was performed to assess the association between age and methylation levels. Specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the three-gene panel as a UTUC biomarker were determined. A receiver operator characteristics (ROC) curve was constructed by plotting the true positive (sensitivity) against the false-positive (1-specificity) rate, and the area under de curve (AUC) was calculated. Differences in proportions test was performed to compare the diagnostic performance of methylation analysis compared to urine cytology. Disease-specific survival curves (Kaplan–Meier with log rank test) were computed for standard variables (tumour stage and grade) and for categorised methylation status. A Cox-regression model comprising all significant variables (multivariable model) was computed to assess the relative contribution of each variable to the follow-up status. For multivariable testing, pathological stage and age were recoded into two groups (Ta–T1 versus T2–4; lower than 75 years versus higher than 75 years). Two-tailed *P*-values were derived from statistical tests, using a computer-assisted programme (SPSS Version 20.0, Chicago, IL), and results were considered statistically significant at $P < 0.05$, with Bonferroni's correction for multiple tests, when applicable.

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3. Results

3.1. Methylation analysis of UTUC tissues and performance of the methylation panel in urines

Most UTUC tissue samples were methylated at *GDF15*, *TMEFF2* and *VIM* promoters, and relative methylation levels were significantly higher when compared to normal mucosas ($P = 0.022$; $P < 0.001$; $P < 0.001$, respectively) (Fig. 1, Supplementary Table 1). Remarkably, the three-gene panel allowed for discrimination of UTUC from normal mucosa with 100% sensitivity and specificity, providing full diagnostic coverage (Table 3). These results were confirmed by ROC curve analysis, with an AUC of 1.000 [95% confidence interval (CI): 0.998–1.000, $P < 0.001$] (Fig. 2B).

The three-gene panel was then tested in a set of urine sediments from 22 UTUC patients and a mix of 20 subjects not carrying UTUC (controls). Methylation levels in UTUC urines were significantly higher than those of controls, for all genes (Supplementary Table 2). Notably, the gene panel identified UTUC in urine with 91% sensitivity and 100% specificity. ROC curve analysis depicted an AUC of 0.923 [95% CI: 0.819–1.000, $P < 0.001$] (Fig. 2C).

To ascertain the clinical usefulness of the methylation assay, its performance was compared with cytological diagnosis performed by an experienced cytopathologist, in 19 cases. Remarkably, the proportion of true positive cases using the panel of methylated genes was significantly higher than that of cytology ($P < 0.001$; Fig. 3). Of 19 cases analysed, only five were cytologically diagnosed as malignant, six as negative for malignancy, and eight were rendered as 'inconclusive/suspicious for malignancy', corresponding to an overall sensitivity of only 26% (Supplementary Table 3). Strikingly, the gene panel correctly identified 17 cases as malignant (89% sensitivity), whereas the remaining two cases were also cytologically diagnosed as negative.

3.2. Association between quantitative promoter methylation and clinicopathological parameters and survival analyses

No association was disclosed between *GDF15*, *TMEFF2* and *VIM* promoter methylation and patient's gender or pathological stage. A significant association was found for *GDF15* methylation levels and age in UTUC patients ($R = 0.303$, $P = 0.022$), but not in controls. Additionally, a significant association of *VIM*

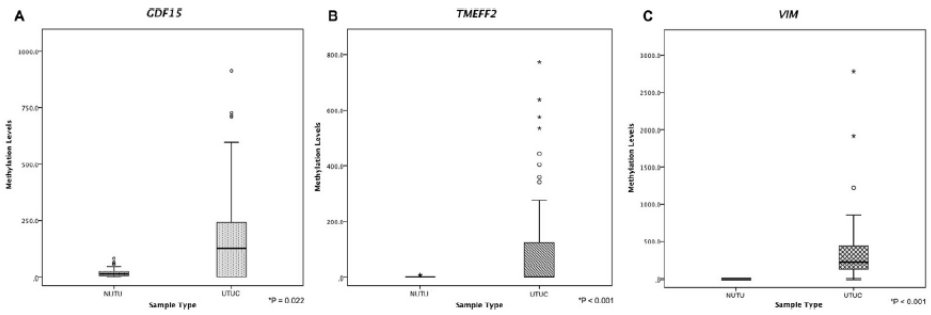

Fig. 1. Box-plots of (A) *GDF15*, (B) *TMEFF2* and (C) *VIM* promoter methylation levels across both normal upper tract urothelium (NUTU) and upper tract urothelial carcinoma (UTUC) tissues (*Mann–Whitney *U* Test).

Table 3
Performance of epigenetic biomarkers for the detection of upper urinary tract urothelial carcinoma in tissue and urine sediments.

	Sensitivity % (*n* positive/*n* total)	Specificity % (*n* negative/*n* total)	PPV (%)	NPV (%)	Accuracy (%)
Tissue samples					
VIM	95 (54/57)	100 (36/36)	100	92	97
VIM/*GDF15*	98 (56/57)	100 (36/36)	100	97	99
VIM/*GDF15*/*TMEFF2*	100 (57/57)	100 (36/36)	100	100	100
Urine samples					
VIM	82 (18/22)	100 (20/20)	100	83	91
VIM/*GDF15*	91 (20/22)	100 (20/20)	100	91	95
VIM/*GDF15*/*TMEFF2*	91 (20/22)	100 (20/20)	100	91	95

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

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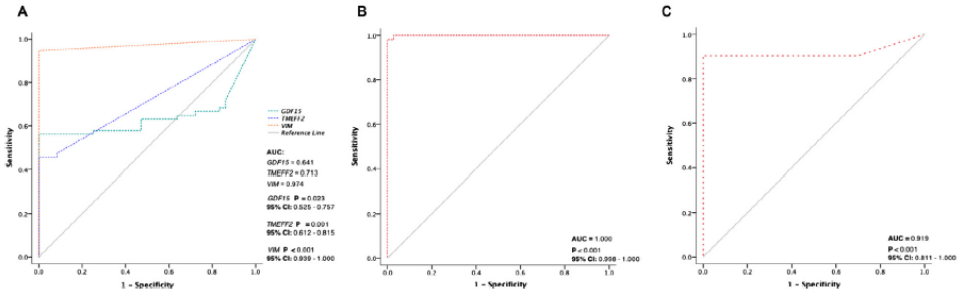

Fig. 2. Receiver operating characteristic curve in upper tract urothelial carcinoma (UTUC) for each individual gene (*GDF15*, *TMEFF2* and *VIM*) in tissues (A), and the combination of three genes (*GDF15*, *TMEFF2* and *VIM*) in tissues (B) and in urines (C).

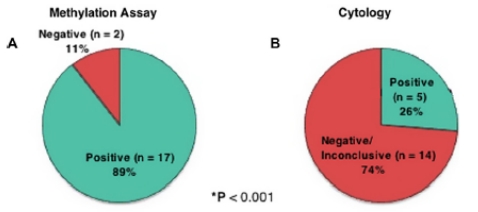

Fig. 3. Pie charts representing the diagnostic test sensitivity for the methylated gene panel – *GDF15*, *TMEFF2* and *VIM* (A) and cytology (B), performed in urine samples from the same patients ($n = 19$) diagnosed with upper tract urothelial carcinoma (*Differences in Proportions Test).

methylation levels and higher tumour grade ($P = 0.015$) was found, and *VIM* methylation levels differed between papillary high-grade and invasive UTUC ($P = 0.006$).

Of the 57 UTUC patients one was lost for follow-up. The median follow-up was 31 months (range: 1–146 months). At the time of the last follow-up, 23 patients were alive with no evidence of cancer, 9 patients were alive with cancer progression and 24 patients had deceased, 19 of which were due to UTUC. For the purpose of survival analyses, all cases were coded based on gene methylation levels using the median value as cutoff. Disease-specific survival analysis showed that patients with low *VIM* promoter methylation levels had a significantly shorter overall survival ($P = 0.013$; Fig. 4C), and a 3.3-fold increased hazard ratio ($P = 0.019$; Table 4). A poor outcome was depicted for patients with higher grade and pathological stage (Fig. 4A and B; $P = 0.03$ and $P = 0.04$, respectively). In univariate Cox-regression analysis, both histopathological type and pathological stage, but not age or gender, carried prognostic information ($P = 0.025$ and $P = 0.049$; Table 4). When clinical and epigenetic variables were introduced in a Cox-regression model for disease-specific survival, pathological stage and *VIM* methylation status were selected in the final model as independent predictors of outcome. After patients were categorised according to pathological stage, low *VIM* methylation levels carried a 18-fold increased risk of cancer-related death (95% CI: 3.24–99.25, $P < 0.001$) in the advanced disease group (pT2-4). Disease-free survival analysis did not disclose any significant association.

4. Discussion

UTUCs are clinically aggressive neoplasms (60% are invasive at diagnosis, compared to 15–20% for bladder UCC), mostly due to late diagnosis, as UTUC is usually

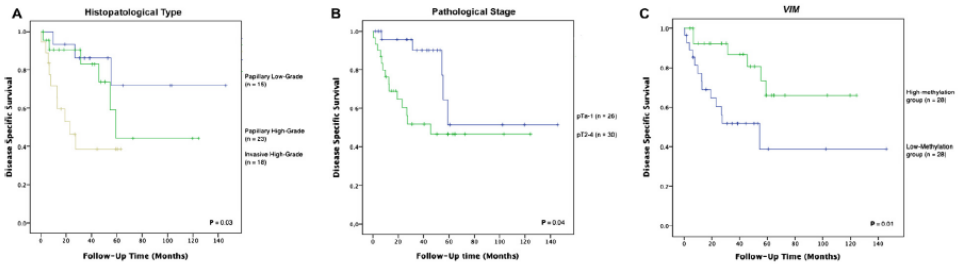

Fig. 4. Disease-specific survival according to clinicopathological parameters [histopathological classification (A) and pathological stage (B)] and *VIM* promoter methylation status (C).

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Table 4
Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of disease-specific survival for 56 upper tract urothelial carcinoma (UTUC) patients (PLG, papillary low-grade; PHG, papillary high-grade and IHG, invasive high-grade).

Disease-specific Survival	Variables	Hazard ratio (HR)	95% CI for OR	*P*
Univariate	**pTNM**	2.81	(1.01–7.83)	**0.049**
	Grade	1.79	(0.45–7.17)	0.411
	PLG versus PHG			
	PLG versus IHG	4.41	(1.21–16.11)	**0.025**
	Age	1.93	(0.69–5.40)	0.208
	Gender	0.36	(0.11–1.23)	0.104
	***VIM* methylation ⩽ median**	3.26	(1.22–8.73)	**0.019**
Multivariate	**pTNM**	3.78	(1.18–12.07)	**0.025**
	Grade	2.51	(0.60–10.56)	0.209
	PLG versus PHG			
	PLG versus IHG	3.02	(0.79–11.53)	0.106
	***VIM* methylation ⩽ median**	5.97	(1.81–19.64)	**0.003**

Significance for bold values: Results were considered statistically significant at $P < 0.05$.

silent at its earliest stages and difficult to detect using ultrasonography and urine cytology. Thus, the development of novel, cost-effective and non-invasive methods for accurate diagnosis of UTUC are likely to have a significant impact in patient management. Gene promoter methylation, a cancer hallmark, has shown promise for accurate detection of urological cancers (especially those of the prostate and bladder) and may thus be of clinical usefulness [15]. We have recently identified a panel of markers (*GDF15*, *TMEFF2* and *VIM* methylation), which detects bladder UCC with high sensitivity and specificity, both in tissue and urine [10]. Owing to the morphological and genomic similarities with UTUC [11], we hypothesised that the same gene panel might show a comparable performance.

Remarkably, this panel identified UTUC with 100% and 91% sensitivity in tissue and urine samples, respectively, maintaining perfect specificity in both. These results constitute a significant step forward in the detection of UTUC and provide a basis for the development of an assay to be used in clinical practice. In fact, owing to the difficult accessibility of the UUT, current methods used to detect a neoplasm in this anatomical location are mostly ineffective. Indeed, our study demonstrates that urine cytology may hardly be considered a diagnostic modality since it only allowed for the diagnosis of 26% of cases. Although higher sensitivity has been reported elsewhere, reaching 40% [16], cases diagnosed by cytology are usually muscle-invasive, thus carrying an unfavorable prognosis [16,17]. On the other hand, high specificity is also very important because most of individuals to which this test might be offered are not carriers of UTUC, and, thus, the requirement for other procedures to confirm an abnormal result of the test, most of which would be invasive, has to be minimised. Finally, it should be emphasised that this gene panel is able to discriminate UCC from renal cell carcinoma, confirming our previous observations [18], thus providing valuable information for cases in which ultrasonography is unable to discriminate tumours of renal pelvic from renal parenchymal origin. The purpose of including renal cell tumours, as well as prostate cancer, as controls in urine samples was to demonstrate the tumour-specificity of the gene panel. Indeed, UTUC, renal cell tumours and prostate carcinoma are amenable to detection in urine samples and occur in similar age spectra. Thus, it would be important for this gene methylation test to accurately predict urothelial carcinoma, discriminating from other common tumours of the genitourinary tract.

Interestingly, survival analysis showed that low *VIM* promoter methylation levels independently predict poor disease-specific survival in UTUC patients, particularly those with high-stage disease. Thus, the gene panel is able to convey not only diagnostic, but also, prognostically important information. Because detection of UTUC based on *GDF15*, *TMEFF2* and *VIM* relies on the identification of higher promoter methylation levels in UCC compared to normal urothelium, the association of low *VIM* promoter methylation levels with worse prognosis might seem paradoxical. However, it should be recalled that *VIM* encodes for vimentin, an intermediate filament, characteristic of cells with mesenchymal phenotype, not expressed in most normal epithelia (including urothelium) nor in most carcinomas [19]. However, *VIM de novo* expression or overexpression has been reported in various epithelial cancers, including those of prostate [20], breast [21] and lung [22], associating with increased tumour growth, invasion and poor prognosis. In these instances, vimentin expression has been associated with epithelial to mesenchymal transition (EMT), a biological process that is associated with an invasive phenotype [19]. Thus, we speculate that during early UUT carcinogenesis, the *VIM* promoter is progressively methylated and the gene is kept silenced, as in normal urothelium, whereas in a subset of UTUC, methylation is decreased, allowing for

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aberrant vimentin expression, due to stimuli leading to EMT. As a consequence, these tumours would be more prone to local invasion and systemic dissemination, thus fostering disease progression and reducing disease-specific survival.

One of the major limitations of our study consists on the relatively small number of cases analysed. However, it should be noted that UTUC is a relatively uncommon neoplasm and we report on the casuistic of a single-centre. This also justifies the fact that in only 19 cases we had information on the cytological diagnosis, which corresponds to the cases diagnosed more recently during the collection period of this study. Thus, a larger and, ideally, multicentre study, should be performed to validate our findings. Moreover, this gene panel is unable to discriminate upper from lower urinary tract UCC. Thus, only after the exclusion of bladder and urethral UCC should a diagnosis of UTUC be considered following a positive test. However, owing to the similarities between UTUC and lower urinary tract UCC, the identification of specific markers is difficult. Some studies proposed that microsatellite instability [23], *CDH1* [24], and *FXYD3* gene expression [25] might provide such discrimination but none has been replicated by other research teams, yet. Thus, positive testing would require additional work-up of the urinary tract.

Summarising, *GDF15*, *TMEFF2* and *VIM* promoter methylation accurately discriminates UTUC from normal urothelium, prostate and renal cell carcinoma, in tissue and urines. Furthermore, low *VIM* methylation levels predict poor prognosis, especially in high-stage disease. Thus, this gene panel may assist in the clinical management of UTUC patients.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2013.08.025.

References

[1] Eble JL, Sauter G, Epstein JI, Sesterhenn IA. Pathology and genetics of tumours of the urinary system and male genital organs: WHO classification of tumours. World Health Organization; 2004.
[2] Munoz JJ, Ellison LM. Upper tract urothelial neoplasms: incidence and survival during the last 2 decades. J Urol 2000;164:1523–5.
[3] Ploeg M, Aben KK, Kiemeney LA. The present and future burden of urinary bladder cancer in the world. World J Urol 2009;27:289–93.
[4] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;62:10–29.
[5] Margulis V, Shariat SF, Matin SF, et al. Outcomes of radical nephroureterectomy: a series from the Upper Tract Urothelial Carcinoma Collaboration. Cancer 2009;115:1224–33.
[6] Roupret M, Babjuk M, Comperat E, et al. European guidelines on upper tract urothelial carcinomas: 2013 update. Eur Urol 2013;63:1059–71.
[7] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.
[8] Shi H, Wang MX, Caldwell CW. CpG islands: their potential as biomarkers for cancer. Expert Rev Mol Diagn 2007;7:519–31.
[9] Jeronimo C, Henrique R. Epigenetic biomarkers in urological tumors: a systematic review. Cancer Lett 2011, Dec 23. [Epub ahead of print].
[10] Costa VL, Henrique R, Danielsen SA, et al. Three epigenetic biomarkers, GDF15, TMEFF2, and VIM, accurately predict bladder cancer from DNA-based analyses of urine samples. Clin Cancer Res 2010;16:5842–51.
[11] Zhang Z, Furge KA, Yang XJ, Teh BT, Hansel DE. Comparative gene expression profiling analysis of urothelial carcinoma of the renal pelvis and bladder. BMC Med Genomics 2010;3:58.
[12] Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. American Joint Committee on Cancer: cancer staging manual. 7th ed. Philadelphia: Lippincott-Raven Publishers; 2010.
[13] Pearson H, Stirling D. DNA extraction from tissue. Methods Mol Biol 2003;226:33–4.
[14] Jeronimo C, Henrique R, Hoque MO, et al. Quantitative RARbeta2 hypermethylation: a promising prostate cancer marker. Clin Cancer Res 2004;10:4010–4.
[15] Phe V, Cussenot O, Roupret M. Interest of methylated genes as biomarkers in urothelial cell carcinomas of the urinary tract. BJU Int 2009;104:896–901.
[16] Messer J, Shariat SF, Brien JC, et al. Urinary cytology has a poor performance for predicting invasive or high-grade upper-tract urothelial carcinoma. BJU Int 2011;108:701–5.
[17] Brien JC, Shariat SF, Herman MP, et al. Preoperative hydronephrosis, ureteroscopic biopsy grade and urinary cytology can improve prediction of advanced upper tract urothelial carcinoma. J Urol 2010;184:69–73.
[18] Costa VL, Henrique R, Ribeiro FR, et al. Quantitative promoter methylation analysis of multiple cancer-related genes in renal cell tumors. BMC Cancer 2007;7:133.
[19] Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci 2011;68:3033–46.
[20] Singh S, Sadacharan S, Su S, Belldegrun A, Persad S, Singh G. Overexpression of vimentin: role in the invasive phenotype in an

Please cite this article in press as: Monteiro-Reis S. et al., Accurate detection of upper tract urothelial carcinoma in tissue and urine by means of quantitative *GDF15*, *TMEFF2* and *VIM* promoter methylation, *Eur J Cancer* (2013), http://dx.doi.org/10.1016/j.ejca.2013.08.025

androgen-independent model of prostate cancer. Cancer Res 2003;63:2306–11.
[21] Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M. Vimentin and epithelial–mesenchymal transition in human breast cancer – observations in vitro and in vivo. Cells Tissues Organs 2007;185:191–203.
[22] Al-Saad S, Al-Shibli K, Donnem T, Persson M, Bremnes RM, Busund LT. The prognostic impact of NF-kappaB p105, vimentin, E-cadherin and Par6 expression in epithelial and stromal compartment in non-small-cell lung cancer. Br J Cancer 2008;99:1476–83.
[23] Roupret M, Fromont G, Azzouzi AR, et al. Microsatellite instability as predictor of survival in patients with invasive upper urinary tract transitional cell carcinoma. Urology 2005;65:1233–7.
[24] Eltz S, Comperat E, Cussenot O, Roupret M. Molecular and histological markers in urothelial carcinomas of the upper urinary tract. BJU Int 2008;102:532–5.
[25] Zhang Z, Pang ST, Kasper KA, et al. FXYD3: a promising biomarker for urothelial carcinoma. Biomark Insights 2011;6:17–26.

Please cite this article in press as: Monteiro-Reis S. et al., Accurate detection of upper tract urothelial carcinoma in tissue and urine by means of quantitative *GDF15*, *TMEFF2* and *VIM* promoter methylation, *Eur J Cancer* (2013), http://dx.doi.org/10.1016/j.ejca.2013.08.025